The evaluation of a phytogenic feed additive as an alternative to antimicrobial growth promoters in broiler feeds

by

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Declaration

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Summary

The use of antimicrobial growth promoters (AGPs) became extremely popular in livestock production after it was discovered that antimicrobials could promote health and animal productivity simultaneously. The overuse and misuse of antimicrobials quickly led to increased concerns over antimicrobial resistance, its potential transmission to humans, and the subsequent threat to public health; and in 2006 the use of antimicrobials as AGPs in animal production was banned by the European Union. Since then, much attention has been focused on finding potential alternatives that have the same positive effects as AGPs, without inducing resistance in micro-organisms. Plant extracts or phytogenics have become a prominent feed additive category that has received much consideration for their potential to replace AGPs. Their bio-active components are known to have antimicrobial, antifungal, antiviral, antioxidant, and/or anticoccidial properties, which all promote gut health by beneficially modulating GIT microflora and by controlling potential pathogenic micro-organisms. Propolis, a resin produced by honey bees, is a prime example of a phytogenic. This study investigated the potential of the phytogenic feed additive, VivoCare®, as an AGP alternative on production parameters, organ and intestinal parameters, carcass and meat characteristics, and the sensory profile of COBB 500 broiler chickens. The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd, and contains caffeoylquinic acid and prodelphinidin bioflavonoids as bio-active components originally identified from propolis extracts.

The primary trial consisted of five experimental diets, each replicated six times. The diets were all fed in three phases (i.e. starter, grower and finisher) and the trial ran for 35 days from hatch to slaughter. The treatments consisted of a negative control containing no AGP (NEG); a positive control containing the commercial AGP Zinc Bacitracin (POS), and three test diets with inclusion levels of 500, 600 and 800 mg of VivoCare® per kg of feed (P500, P600, P800). A secondary trial was conducted to investigate the effect of VivoCare® on skeletal parameters. This trial was performed in the same housing system with the same experimental diets, but with 10 measurements from one cage per treatment.

Results from this study showed that the negative control, positive control and three VivoCare® test diets all performed equally well in terms of growth performance. Production parameters that were investigated include: average live weight gain, average feed intake, feed conversion ratio (FCR), liveability, average daily gain (ADG), protein efficiency ratio (PER) and European production efficiency factor (EPEF). The absence of significant gizzard erosion in VivoCare® fed birds confirmed that the product was non-toxic and safe to use as a feed additive. With regards to the organ and intestinal parameters, significant overall differences were also absent between the VivoCare® diets and the two control groups. Organ and intestinal parameters that were measured include: organ relative weights, intestinal pH and liver colour. VivoCare® thus had no significant effects on gut health and immune status in this trial. The negative control did show slightly more evidence of exposure to immunological stress, however, differences were not prominent and further research was recommended to support these results. The VivoCare® diets also had no significant effects on the carcass quality, meat quality and skeletal parameters. This was seen in comparison to the negative control, whereby VivoCare® had statistically similar results for carcass weights; dressing percentage; carcass portion and breast component yields; breast and thigh pH; breast meat colour; tibia bone weight, length, diameter, breaking

strength, fat free dry weight, ash percentage and mineral content; thaw loss and cooking losses. Correlation tests indicated that heavier bones could be associated with longer, thicker and stronger measurements; however, it was seen that bones that were thicker were not also necessarily stronger. Significant effects of VivoCare® on the descriptive sensory analysis (flavour, aroma and texture) of fillets from the trials were also absent. Results from the sensory profile did, however, indicate that the wet-feather/sweaty/barnyard aroma was significantly more prominent in the negative control meat in comparison to the positive control meat. It was speculated that this off-odour was as a result of a volatile organic compound (VOC), such as 4-ethylphenol, which may have been more prominent in the negative control due to oxidative processes.

Overall, the VivoCare® product may have promising potential as an alternative for AGPs, as it did not bring about any negative results throughout this study and it performed at a statistically similar level to the positive (AGP-included) control. A possible reason for the numerous statistical similarities observed in this study could have been due to the birds being raised in an optimal environment that was reasonably stress- and pathogen-free; and AGPs have been shown to lack growth-promoting effects in optimal living conditions. Further research is thus recommended to investigate the effects of VivoCare® in sub-optimal circumstances (i.e. under the influence of an intentional stressor) or at different inclusion levels, so as to evaluate its full potential and capabilities as a potential alternative to AGPs.

Additional measurements and techniques that are recommended for future studies include: histomorphology studies of the GIT; investigation of blood constituents (i.e. lipid concentrations in the serum and antibody titer); evaluation of carcass fat content, bone density, mineral digestibility, and cortical and trabecular bone thickness; and methods to analyse and compare VOCs and fatty acid concentrations, as well as, meat and lipid oxidative rates.

Opsomming

Die gebruik van antimikrobiese groeibevorderers (AGPs) het baie gewild geword in lewendehawe produksie nadat dit ontdek is dat antimikrobiese middels gelyktydig gesondheids- en dierproduktiwiteit kan bevorder. Die oorbenutting en misbruik van antimikrobiese middels het vinnig gelei tot groter bekommernisse oor antimikrobiese weerstand, die potensiële oordrag na die mens en die daaropvolgende bedreiging vir die volksgesondheid; en in 2006 is die gebruik van antimikrobiese middels as AGP's in dierproduksie verban deur die Europese Unie. Sedertdien is baie aandag gevestig op die vind van potensiële alternatiewe wat dieselfde positiewe effekte as AGP's het, sonder om weerstand in mikroörganismes te veroorsaak. Plant ekstrakte of fitogenika het 'n prominente toevoegingskategorie geword, wat baie oorweging gekry het vir hul potensiaal om AGP's te vervang. Hul bio-aktiewe komponente is bekend om antimikrobiese, antifungale, antivirale, antioksidante en / of anticoccidiale eienskappe te hê, wat almal dermgesondheid bevorder deur SVK-mikroflora positief te moduleer en deur potensiële patogene mikroörganismes te beheer. Propolis, 'n hars wat deur heuningbye geproduseer word, is 'n uitstekende voorbeeld van 'n fitogeniese AGP. Hierdie studie ondersoek die potensiaal van die fitogene bymiddel, VivoCare®, as 'n AGP-alternatief vir produksiesparameters, orgaan- en dermparameters, karkas en vleiskarakteristieke, en die sensoriese profiel van COBB 500 braaikuikens. Die VivoCare® produk is vervaardig deur Beonics Feed Supplements (Edms) Bpk, en bevat kaffeoilchinsuur en prodelfinidien bioflavonoïede as bio-aktiewe komponente wat oorspronklik uit propolis ekstrakte geïdentifiseer is.

Die primêre ondersoek het bestaan uit vyf eksperimentele diëte, elk ses keer herhaal. Die diëte is almal gevoer in drie fases (dit wil sê aanvangs-, groei- en afrondingsdieet) en die proef het 35 dae van uitbroei tot by slag geneem. Die behandelings het bestaan uit 'n negatiewe kontrole wat geen AGP (NEG) bevat nie; 'n positiewe kontrole wat die kommersiële AGP-sinkbasitrasien (POS) bevat, en drie toetsdiëte met insluiting van 500, 600 en 800 mg VivoCare® per kg voer (P500, P600, P800). 'n Sekondêre proef is uitgevoer om die effek van VivoCare® op skeletparameters te ondersoek. Hierdie proef is uitgevoer in dieselfde behuisingstelsel met dieselfde eksperimentele diëte, maar met 10 metings van een hok per behandeling.

Resultate van hierdie studie het getoon dat die negatiewe kontrole, positiewe kontrole en drie VivoCare®-diëte almal ewe goed presteer het ten opsigte van groeiprestasie. Produksieparameters wat ondersoek is, sluit in: gemiddelde voerinname, voeromsetverhouding (FCR), oorlewingstempo, gemiddelde daaglikse toename (ADG), proteïen doeltreffendheidsverhouding (PER) en Europese produksie doeltreffendheidsfaktor (EPEF). Die afwesigheid van beduidende maalmaag erosie in VivoCare® gevoerde voëls het bevestig dat die produk nie-giftig is en veilig as 'n voerbymiddel gebruik kan word. Met betrekking tot die orgaan- en dermparameters was beduidende verskille tussen die VivoCare®-dieet en die twee proefgroepe afwesig. Orgaan- en dermparameters wat gemeet is, sluit in: orgaan relatiewe gewigte, derm pH en lewer kleur. VivoCare® het dus geen beduidende effekte gehad op derm gesondheid en immuunstatus in hierdie proef nie. Die negatiewe kontrole toon effens meer bewyse van blootstelling aan immunologiese stres, maar verskille was nie prominent nie en verdere navorsing is aanbeveel om hierdie resultate te ondersteun. Die VivoCare®-dieet het ook geen beduidende effekte op die karkasgehalte, vleiskwaliteit en skeletparameters gehad nie. Dit is gesien in vergelyking met die negatiewe kontrole, waarvolgens VivoCare® statisties soortgelyke resultate vir

karkasgewigte gehad het; uitslagpersentasie; karkas snit opbrengste en bors weefsel komponente opbrengste; bors en dy pH; borsvleis kleur; tibia been gewig, lengte, deursnee, breeksterkte, vetvrye droë gewig, as persentasie en minerale inhoud; ontdooi verlies en kook verliese. Korrelasietoetse het aangedui dat swaarder bene geassosieer kan word met langer, dikker en sterker metings; Daar is egter gesien dat bene wat dikker was, nie noodwendig sterker was nie. Betekenisvolle effekte van VivoCare ® op die beskrywende sensoriese analise (geur, aroma en tekstuur) van filette van die proewe was ook afwesig. Resultate van die sensoriese profiel het egter aangedui dat die natveer/sweet/skuur aroma beduidend meer prominent in die negatiewe kontrole vleis was in vergelyking met die positiewe kontrole vleis. Daar is gespekuleer dat hierdie reuk was as gevolg van 'n vlugtige organiese verbinding (VOC), soos 4-etielfenol, wat meer prominent in die negatiewe kontrole kon wees as gevolg van oksidatiewe prosesse.

Oor die algemeen kan die VivoCare®-produk belowende potensiaal as alternatief vir AGP's hê, aangesien dit nie negatiewe resultate gedurende hierdie studie tot gevolg gehad het nie en dit op 'n statisties soortgelyke vlak uitgevoer op die positiewe (AGP-ingesluit) beheer. 'n Moontlike rede vir die talle statistiese ooreenkomste wat in hierdie studie waargeneem word, kon gewees het as gevolg van die feit dat die voëls in 'n optimale omgewing wat redelik stres- en patogeenvry was geproduseer is; en AGP's het getoon dat hulle groei bevorderende effekte in optimale lewensomstandighede het. Verdere navorsing word dus aanbeveel om die effekte van VivoCare® in suboptimale omstandighede (d.w.s onder die invloed van 'n daging) of op verskillende insluitingvlakke te ondersoek, ten einde die volle potensiaal en vermoëns daarvan as 'n potensiële alternatief vir AGP's te evalueer.

Bykomende metings en tegnieke wat aanbeveel word vir toekomstige studies sluit in: histomorfologiese studies van die SVK; ondersoek van bloedbestanddele (d.w.s lipiedkonsentrasies in die serum en teenliggaamtiter); evaluering van karkasvetinhoud, beendigtheid, minerale verteerbaarheid en kortikale en trabekulêre beendikte; en metodes om VOCs en vetsuurkonsentrasies te analiseer en te vergelyk, asook vleis en lipied oksidatiewe dosisse.

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Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has been unavoidable.

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List of abbreviations

a*	Redness
ADG	Average daily gain
AGP	Antimicrobial growth promoter
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
b*	Yellowness
BRICS	Brazil, Russia, India, China and South Africa
BW	Body weight
BWG	Body weight gain
Ca:P	Calcium to phosphorus ratio
CAPE	Caffeic acid phenethyl ester
CP	Crude protein
DFD	Dark, firm and dry
DSA	Descriptive sensory analysis
EPEF	European production efficiency factor
EU	European Union
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastrointestinal tract
GLM	General linear model
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
L*	Lightness
M	Molar
min	Minutes
NDOH	National Department of Health
%	Percentage
PA	Proanthocyanidin
PCA	Principle component analysis
PD	Prodelphinidin
PER	Protein efficiency ratio
PFA	Phytogenic feed additive
pH_I	Initial pH

pH_U	Ultimate pH
PSE	Pale, soft and exudative
r	Pearson's correlation coefficient
SAASP	South African Antibiotic Stewardship Programme
SAPA	South African Poultry Association
TD	Tibial dyschondroplasia
WHA	World Health Assembly
WHO	World Health Organisation
UV	Ultraviolet
VOC	Volatile organic compound
WHC	Water holding capacity

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Chapter 1

Introduction

The discovery and usage of antimicrobials in human medicine was followed closely by its use in livestock production. Indications of improved production efficiency through the use of antimicrobials was reported as early as 1946 by Moore *et al.*, whose results showed that the inclusion of antimicrobials in feed fed to chickens caused an increase in weight gain (Moore *et al.*, 1946). A growing and avid interest in the beneficial effects of antimicrobials on livestock performance soon followed, with attention turning to include other animal species, such as pigs and cattle (Fountaine & Atkeson, 1950; Jukes *et al.*, 1950). The 1950s represented the post-war period when the demand for food and animal protein was rapidly increasing in the United States and Europe. With the then realized potential benefits of antimicrobials in promoting both disease prevention and growth efficiency simultaneously, antimicrobials soon became a fundamental part of a new agricultural production model with the inevitable movement towards their inclusion in feeding programs (Laxminarayan *et al.*, 2015). It was at around this time too that evidence of antimicrobial resistance came to light (Starr & Reynolds, 1951); however, the favourable effects of antimicrobial use on livestock production and its subsequent contribution to a reduction in meat prices during the 1950s, far outweighed the possible risks that had been noted at that time (Laxminarayan *et al.*, 2015).

A recommendation by Professor Swann and his colleagues was suggested as early as 1969 in a report to the British Parliament, to ban the sub-therapeutic use of antimicrobials in animal feeds due to concerns regarding the development of antimicrobial resistance of pathogens in humans (Swann *et al.*, 1969). Several reports with similar recommendations followed, yet in spite of the early concerns and warnings, the prevalent use of antimicrobials generated a selection pressure which promoted the spread and development of pathogen resistance worldwide (Laxminarayan *et al.*, 2015).

Antimicrobial resistant genes and microbes can pass between humans, animals, food, water and the environment and their transmission is further facilitated by trade, travel, and human and animal migration (World Health Organisation, 2015). Since there are a number of antimicrobials which are used in both livestock production and human medicine, the transmission of organisms with antimicrobial resistance (AMR) to humans poses a severe threat to public health. This threat is particularly severe in low- and middle-income countries, such as the so called BRICS nation (Brazil, Russia, India, China and South Africa), where the overuse and misuse of antimicrobials is excessive (den Hartog *et al.*, 2016).

Sweden was the first nation to ban the use of sub-therapeutic levels of antimicrobials as AGPs in animal feeds in 1986 (Wierup, 2001); and by 2006, the practice was banned altogether by the European Union (EU) (Regulation 1831/2003/EC on additives for use in animal nutrition) (European Commission, 2005). Since then, the search for alternative options and novel approaches for the replacement of AGPs has received much attention by researchers in the 21st century. The relatively new term, phytogenics, refers to a category of plant-derived substances or extracts which have the potential to replace AGPs. Phytogenics consist of bio-active components, which promote growth and immune status through their antimicrobial, antifungal, antiviral,

antioxidant, and/or anticoccidial effects (Murugesan *et al.*, 2015). A prominent example of a phytogenic, is the resinous substance produced by honey bees, known as propolis.

VivoCare® is a newly developed phytogenic product that was produced by Beonics Feed Supplements (Pty) Ltd, after studying signal molecules and gene expression in different types of propolis, and it contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components. Studies have shown that these compounds have antitumor, antioxidant, and antibacterial properties; which thus gives this product its potential to promote animal growth and production (Midorikawa *et al.*, 2001; Plumb *et al.*, 2002; Fujii *et al.*, 2013a; Fujii *et al.*, 2013b; Rodríguez-Pérez *et al.*, 2016).

The aim of this study was to investigate the effectiveness of VivoCare® as an alternative to infeed AGPs in broiler diets. In order to achieve this aim, growth production parameters, organ and intestinal parameters, carcass and meat quality characteristics, and organoleptic attributes, were all taken into account and evaluated. Broilers that were fed VivoCare® supplemented diets were expected to outperform the negative control in terms of production performance (unless the negative and positive control had statistically similar results), in order for this phytogenic to be considered as a successful AGP alternative. VivoCare® was tested against a positive control containing the in-feed AGP, Zinc Bacitracin; as well as against a negative control, containing no additives.

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Chapter 2

Literature review

2.1. Introduction

Antimicrobials have been used across various disciplinary groups including human and animal medicine, plant agriculture, food production and industrial application. With regards to animals for food production, antimicrobials have been typically used in three ways: to cure disease (therapeutic application), to prevent disease (prophylactic application) and as antimicrobial growth promoters (sub-therapeutic concentrations which improve growth and feed efficiency in livestock) (Laxminarayan *et al.*, 2015). The use of antimicrobial growth promoters (AGPs), in particular, became increasingly popular with the shift towards more intensive production techniques so as to meet the growing demand for animal proteins.

There are at least two ways in which AGPs can enhance farm productivity, namely; by improving growth rates and feed efficiency of livestock (Gaskins *et al.*, 2002; Dibner & Richards, 2005) and by acting as a substitute for hygiene-management practices with the potential to improve labour and capital productivity in animal housing systems and during transportation (Key & Mcbride, 2014; Laxminarayan *et al.*, 2015). Antimicrobial growth promoters have also been used to minimize product variation in terms of weight and size, subsequently reducing financial costs and penalties for animals which fall outside the defined range required for mechanical processing and market acceptance (Liu *et al.*, 2005).

In spite of the extensive use of AGPs globally, definitive conclusions are still lacking on their exact effects on productivity. Factors including the species, age and genetic potential of the animal, as well as the management and hygienic conditions, can all contribute variably to the effectiveness of sub-therapeutic AGPs on growth response (Wierup, 2001). It has also been shown that AGPs do not have growth-promoting effects in “germ-free” animals (Coates *et al.*, 1955, 1963).

Concerns relating to the development and transmission of antimicrobial resistant genes and microbes to humans via animal products, and the subsequent threat to public health, eventually lead to the complete ban of AGP use in animal production by the European Union in 2006 (Regulation 1831/2003/EC on additives for use in animal nutrition) (European Commission, 2005). The search for effective and feasible alternatives to AGPs has been ongoing ever since.

Phytogenic feed additives (PFAs) are also known as natural growth promoters, due to their proven abilities to acts as AGP alternatives. Much literature has been published on the successes of different PFAs in promoting growth and immune status in animal production. The objective of this review was to briefly describe the effects of the AGP ban on animal production; with special reference to AGPs and the poultry industry in South Africa; as well as give an overview of a particular PFA known as propolis, some of its relevant bio-active ingredients, and its potential to replace AGPs in broiler nutrition through its effects on growth performance, organ and intestinal parameters, and carcass and sensory attributes.

2.2. The effects of banning AGPs

On a positive note, even though bans may not be actively in place in all parts of the world at present, the use of AGPs in certain livestock sectors has been said to be decreasing; due in part to the growing influence of consumer preference for perceived healthier food products (e.g. organic and AGP-free) (Laxminarayan *et al.*, 2015). In 2003, the McDonald's Corporation proclaimed that it would no longer accept meat products from suppliers who made use of AGPs. Various major food chains and retail companies soon followed suit (MacDonald & Wang, 2011). Producers who rely on export markets are thus being forced to do away with AGPs if they plan to sell to the EU and other like-minded markets (Dibner & Richards, 2005). Although these movements and trends hold great promise for the future, the complete and global eradication of AGP use in animal production could take decades; which means that antimicrobial resistance will still remain a threat for many years to come.

Animal product consumption is one of the predominant means of antimicrobial transmission. It was estimated by Laxminarayan *et al.* (2015) that in 2010 the global consumption of antimicrobials in food production was 63,151 (± 1,560) tonnes; while they predicted that by 2030 this amount will rise by 67% to 105,596 (± 3,605) tonnes. The overuse and misuse of antimicrobials occurs predominantly in low- and middle-income countries, including the so called BRICS nations (Brazil, Russia, India, China and South Africa). Factors contributing to this tendency and intensifying the issue further include high levels of poverty where access to clean water, hygiene and sanitation is limited; the high incidence rate of infectious diseases; the unregulated availability of antimicrobials without prescription over-the-counter and from street-vendors; the lack of awareness and education on the adverse effects of incorrect antimicrobial use on public health; and a limited capacity of pharmaceutical companies to enforce or regulate correct usage (den Hartog *et al.*, 2016). It has been said that the developing world will claim more than 90% of the estimated 10 million AMR-linked deaths per annum by 2050 (O'Neill, 2014). The global predicted AMR-related mortalities per year for 2050 can be seen in Figure 2.1.

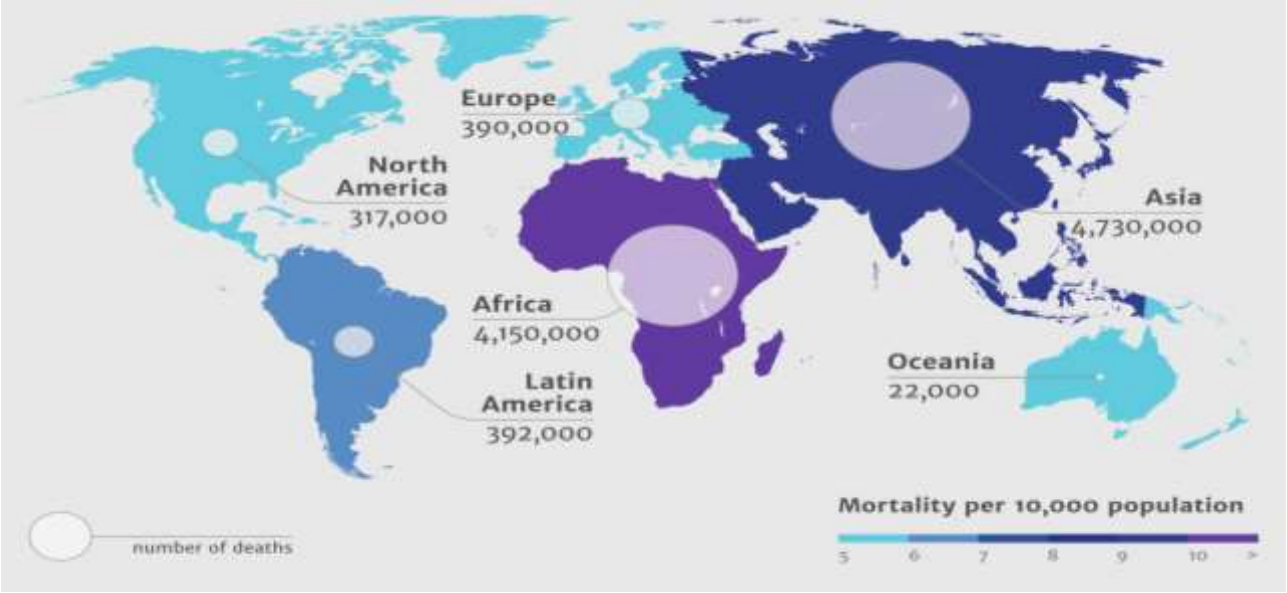

Figure 2.1 The global predicted antimicrobial-resistance-related mortalities per year for 2050 (O'Neill, 2014)

In optimized production systems, the effect of AGPs on growth has appeared to be minimal. The ban of AGPs thus has little effect on the economic status of high-income, developed countries; while lower income countries with less developed hygiene, feed and production practices stand to suffer a considerably more severe blow (Laxminarayan *et al.*, 2015). This presents a lose-lose situation for developing countries, as banning AGPs could risk great economic loss; while continuing the use of AGPs poses an imminent threat to public health. In an article published in 2001, Wierup stated that antimicrobials should principally be used as a last resort instead of as a substitute for agricultural management practices (Wierup, 2001). Antimicrobials are not necessary to promote growth, yet they are vital in treating infectious diseases and maintaining animal health (Laxminarayan *et al.*, 2015).

In 2002, Emborg *et al.* described the effects of the AGP ban on the broiler industry in Denmark. It was reported that both productivity and liveability were not significantly affected by the ban. Feed conversion ratios (FCR) did show a slight increase of 0.016 kg/kg from 1995 to 1999, however they then decreased again from 1999 to 2002. Denmark thus demonstrated that it was possible to eliminate the use of AGPs from farming practices with minimal impacts on production. Denmark was and still is, however, a high-income country with developed and efficient management practices which would have made the transition to AGP-free practices much easier. The above mentioned results are thus more likely an example of a best-case scenario following the ban of AGPs. One can only speculate about worst-case scenarios for less developed countries, who may take decades to adapt their management practices to achieve even remotely similar results to when AGPs were used, which may in turn have a devastating impact on their economy.

2.3. AGPs and the poultry industry in South Africa

In South Africa, the poultry industry remains the largest sole contributor to the agricultural sector. The South African Poultry Association (SAPA) estimated that in 2016, poultry production claimed approximately 18% of the total agricultural gross value and contributed roughly 39% to the total gross value of animal products (SAPA, 2016).

The South African poultry industry is divided into two predominant groups. Approximately 76% of the birds are used for meat products, while the remaining 24% are used for egg production. From 2004 until 2008 it was seen that the broiler industry in South Africa underwent a considerable growth phase, boasting a 7% average annual increase. This growth period related to the increased demand for products and more stable input costs. From 2009 to 2014, however, industry growth (based on kg meat produced) slowed dramatically to less than 1% per annum. Contributing factors included; increased production costs, a decline in disposable income of consumers and the reduced demand for broiler products produced locally due to the influx of cheaper imported poultry meat products (SAPA, 2015).

Globally, South African poultry producers compare favourably with competitors in terms of production efficiencies. It is the cost of production or more specifically, feed costs, which contribute largely to the reduction of the country's competitiveness. According to the SAPA 2015 Industry Profile, top producing broiler farms in South Africa are achieving feed conversion ratios (FCRs) of approximately 1.61 with performance efficiency

Factor values (EPEF) of around 304. The average age and weight at slaughter is 33.75 days at 1.8 kg, respectively (SAPA, 2015).

In a survey conducted by Eagar *et al.* (2012), it was seen that for the period of 2002 until 2004 as much as 29% of all antimicrobials available in South Africa for livestock production, were in the form of pre-mixes. This and other studies have confirmed that South Africa utilizes large quantities of antimicrobials in livestock for food production; many of which have been banned for use as growth promoters in other countries. The threat of antimicrobial resistance is thus a real and growing problem in South Africa, and its risk to public health is only worsened by the high incidence rate of infectious diseases (especially those of bacterial origin) and the HIV/AIDS epidemic (Moyane *et al.*, 2013).

On the 17th May 2014, the World Health Organisation (WHO) adopted the World Health Assembly's (WHA) resolution WHA67.25 in response to the urgent global call to take action against AMR. In South Africa, the National Department of Health (NDOH) in conjunction with the South African Antibiotic Stewardship Programme (SAASP) responded by hosting the first Antimicrobial Resistance Summit in October 2014. The purpose was to g*et* all relevant stakeholders to commit to the implementation of the Antimicrobial Resistance National Strategy Framework for South Africa which proposed a three year timeframe (2016 – 2019) in which to review antimicrobial use in animal feeds and additives, and then develop an AMR prevention strategy and operational plan while also considering the viability of promising alternatives (Mendelson & Matsoso, 2015).

2.4. Potential alternatives to AGPs

Alternative options and novel approaches for the replacement of AGPs have become a core focus in the 21st century. Antimicrobials are said to improve animal growth and production through various possible modes of action which affect the microbiota composition in the gut and modulate the immune system either directly or indirectly (den Hartog *et al.*, 2016). Some key examples of functional feed ingredients in broiler nutrition which could act as AGP alternatives and initiate similar responses are: organic acids, probiotics, prebiotics and phytogenic plant extracts (den Hartog *et al.*, 2016). In comparison to the more familiar non-antimicrobial growth promoter options such as probiotics and organic acids; phytogenics are a reasonably new category of feed additives that have recently received much attention and consideration for their potential in replacing AGPs.

2.4.1. Phytogenic feed additives

Plants and plant-derived products have been used traditionally for centuries to fight infections and disease. While performing their normal metabolic functions, plants also produce chemical compounds which can be divided haphazardly into two groups, i.e. primary and secondary metabolites. All plants produce primary metabolites which include the main nutrients: proteins, fat, sugars, etc. (Hashemi & Davoodi, 2011). Only a smaller variety of plants produce secondary metabolites, also known as phytochemicals, which although not necessary for the plant's basic function, may assist to protect them against pathogens, predators and other environmental and physiological stresses (Wenk, 2003). It is these secondary compounds; originating primarily from herbs, spices and plants; which are evaluated as phytogenic feed additives for their potential in AGP replacement.

Phytogenic feed additives (PFAs), also known as phytobiotics or natural growth promoters, have been defined in the agricultural sector as plant-derived products which are added to feed to boost livestock productivity and performance, ultimately improving food quality (Windisch *et al.*, 2008). Knowledge on their exact modes of action, application, and possible interactions with other feed additives, is still being widely investigated; as the effect of each particular PFA varies according to their botanical origin, composition and method of processing. It is for this reason that most past studies have tended to examine blends of active compounds and their overall effect on production performance, rather than pinpointing and defining exact physiological impacts (Windisch *et al.*, 2008).

Besides the usual botanical classification, PFAs can be classified according to numerous other characteristics, such as: the portion of plant used (entire plant, stem, root, flower, seed, etc.); the type of plant (herbs, grasses, trees, shrubs, etc.); the climate (tropical, temperate, etc.); their therapeutic value (antimicrobial, antioxidant, immunostimulant, etc.); and the means of administration (tincture, syrup, tisanes, etc.) (Hashemi & Davoodi, 2011). In some literature, authors prefer to categorize PFAs according to their function, namely: as sensory additives (affect food and feed odour, palatability and/or colour); technological additives (act as antioxidants, reduce mycotoxin contamination in feeds, etc.); zootechnical additives (act as immunomodulators, digestive stimulants, non-microbial growth promoters, performance and quality enhancers of animal products, etc.); and nutritional additives (minerals, vitamins, plant enzymes, etc.). A number of phytogenic additives, however, cannot be strictly assigned to one of these specific groups as they bring about more than one of these beneficial effects (Karásková *et al.*, 2015).

Due to the fact that secondary metabolites generally exist in limited quantities within plants, these compounds are often extracted and refined to produce a plant-extract concentrate which can be used as PFAs in smaller amounts with a more prominent effect. Three examples of prominent phytogenic plant extracts include: essential oils (hydro-distilled oil extracts from volatile plant compounds), oleoresins (extracts obtained using non-aqueous solvents), and bioflavonoids (polyphenolic molecules which can be efficiently produced through genetic engineering methods based on multienzyme pathways in plants and microbes) (Ververidis *et al.*, 2007; Windisch *et al.*, 2008).

A prime example of a phytogenic, is the natural, resinous, plant-derived substance that is produced by honey bees, known as propolis. Propolis has a wide range of bio-active ingredients with beneficial properties that have been demonstrated successfully in both humans and animals. It has been these promising results that have inspired further investigation into the use of this substance and its bio-active constituents as natural feed additives for the potential replacement of AGPs.

2.4.2. Propolis

The word propolis is of Greek origin and is derived from the word “pro”, meaning “in favour of”, and “polis”, which refers to a “city”. The word thus matches its purpose in the hive; as propolis is a natural, resinous substance produced and used by honey bees to protect the hive, their larvae and themselves from harmful micro-organisms. Through mastication, bees mix collected exudate from various plant sources with salivary enzymes. This mixture is then combined with beeswax and other compounds to produce the unique propolis

product (Banskota *et al.*, 2001). Bees use propolis to seal and insulate their hives during construction. This helps to maintain an internal temperature of around 35°C; while the antimicrobial and anti-inflammatory activities of this resin aid in protecting hive inhabitants from bacterial, viral and fungal infections (Farooqui & Farooqui, 2012).

Since its popularity in traditional folk medicine, numerous studies have demonstrated the extensive range of beneficial biological and pharmacological activities that propolis exhibits. Some such properties come to include; antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, anticancer, antitumor, antiulcer, hepatoprotective, neuroprotective and cardioprotective actions (Farooqui & Farooqui, 2012). The potential of propolis to benefit human and animal health is thus immense.

2.4.3. Bio-active ingredients of propolis

Propolis resin has a very complex composition with a variety of chemical components including; flavonoids, phenolic acids and their esters, terpenes, fatty acids, aromatic alcohols and aldehydes, stilbenes, and steroids (Akyol *et al.*, 2013). The exact chemical composition of propolis resin is not fixed, but largely dependent on the types of plants from which bees have collected exudates. This means that, although the biological activity of propolis and its action against micro-organisms is always present, the activity of each sample is as a result of a completely different chemical composition which is closely related to the geographical location and climatic zones of exudate collection sites (Bankova, 2005). It is for this reason that authors should provide details on the type of propolis and its active ingredients when submitting publications, so as to allow for adequate future comparisons. This has unfortunately not been the case with many past studies involving propolis as a feed additive; as often only dietary concentrations are supplied as a reference (Mahmoud *et al.*, 2016).

Of the numerous phytochemicals that are known to exist in propolis, bioflavonoids in particular, have encouraged much interest and research into the potential of these compounds in replacing AGPs in animal production due to their wide range of biological and pharmacological actions (Narayana *et al.*, 2001). The more specific, propolis-based, active ingredients of interest in this study include the bioflavonoid, prodelphinidin, and the phenolic compound, caffeoylquinic acid.

2.4.3.1. Bioflavonoids

Bioflavonoids were discovered serendipitously by Dr A. Szent-Györgyi and his colleague Dr. S.T. Rusznyak at around the same time as their discovery of ascorbic acid (more commonly known as vitamin C). In 1936, they found that a pure solution of ascorbic acid was not effective in treating a patient with subcutaneous capillary bleedings; while an impure solution with extracts of lemon juice or Hungarian red peppers achieved rapid success in regulating vascular permeability. They proposed the term “vitamin P” as a name for the group of compounds that brought about this type of effect, whilst also demonstrating that vitamin P and C were synergistic and interdependent (Bentsáth *et al.*, 1936; Rusznyák & Szent-Györgyi, 1936; Passwater, 1994). Since then, the term “Vitamin P” has been replaced with the word flavonoid or bioflavonoid, and extensive research into their classification, biochemical effects and their potential application has followed.

Bioflavonoids have subsequently been defined as a group of plant derived secondary metabolites which are predominantly responsible for the attractive colouring of fruits and flowers. They are commonly found in fruit, vegetables, bark, roots, grains, flowers, stems, tea and wine (Middleton, 1998). Since their discovery in the 1930s, researchers have identified well over 4000 different varieties of flavonoids (Nijveldt *et al.*, 2001). Flavonoids are polyphenolic compounds and can be classified according to their molecular structure as flavonols, flavones, flavonones, flavanols, isoflavones and flavan-3-ols, depending on where particular substituents are positioned on the parent molecule (Narayana *et al.*, 2001).

Bioflavonoids are known to bring about a wide range of beneficial biological effects. Research has shown that they can initiate anti-hepatotoxic, antiallergic, antiviral, anticarcinogenic, anti-inflammatory and anti-ulcerogenic responses (Middleton, 1998; Xiao *et al.*, 2011). They are powerful antioxidants and are known for their free radical scavenging capabilities (Korkina & Afanas'Ev, 1996; Xiao *et al.*, 2011).

2.4.3.2. Pro-delphinidin

Proanthocyanidins (PAs) are polyphenolic compounds which are commonly found in plant-derived foods such as cereals, fruits and beverages; and they are the second most abundant natural phenolics after lignins (Behrens *et al.*, 2003; Teixeira *et al.*, 2016). These condensed tannins are secondary metabolites that are produced by plants under both normal and stressful conditions (e.g. UV radiation, water stress, and bacterial and fungal infections) (Koes *et al.*, 1994; Teixeira *et al.*, 2016). Chemically, they are oligomeric flavonoids and their characteristic structure consists of two phenyl rings and a heterocyclic ring. Depending on the hydroxylation pattern on the phenyl rings, PAs can be divided into propelargonidins, procyanidins and prodelphinidins. Proanthocyanidins with gallocatechin or epigallocatechin as subunits are termed prodelphinidins (PDs) (Gu *et al.*, 2003).

PDs have not been as widely studied as PAs in the past, due to their apparent low abundancy in dietary sources (Teixeira *et al.*, 2016). The absence of commercial standards, appropriate analytical methods for detection and identification, and limited synthesis pathways, were the main contributing factors which hindered research with this compound previously (Makabe, 2013; Teixeira *et al.*, 2016). These challenges are, however, being overcome with progressively more research and articles being published in recent years on the extraction, composition, synthesis, and biological activities of PDs (Fujii *et al.*, 2013a; Fujii, *et al.*, 2013b; Makabe, 2013; Yang *et al.*, 2016).

Authors have reported that PDs have antitumor, antioxidant, and antibacterial properties. Fuji *et al.* (2013a; 2013b) managed to successfully produce synthetic prodelphinidins, where after they demonstrated that these compounds had antitumor effects against human prostate cancer cell lines, indicating that PDs could have potential as chemo-preventing agents (Fujii *et al.*, 2013a; Fujii *et al.*, 2013b). Rodríguez-Pérez *et al.* (2016) demonstrated that PDs had antibacterial properties after they isolated these and other phenolic compounds from a cranberry extract. When tested *in vitro* against uropathogenic strains of *Escherichia coli*, they found that PDs significantly decreased surface hydrophobicity, thus reducing the ability of these bacteria to adhere to host surfaces (Rodríguez-Pérez *et al.*, 2016). In a study by Yang *et al.* (2016), the antioxidant activity and PD

concentration from bayberry leaf hot extracts, were shown to have a positive and linear correlation; while Plumb *et al.* (2002) demonstrated potent antioxidant capabilities of PD dimers from pomegranate peel.

2.4.3.3. Caffeoylquinic acids

Caffeoylquinic acids are phenolic acids which are esters of polyphenolic caffeic acid and quinic acid. Caffeic acid is classified as a hydroxycinnamic acid that consists of both phenolic and acrylic functional groups and is a crucial intermediate in the biosynthesis of lignin (Gowri *et al.*, 1991). Tatefuji *et al.* (1996) were the first to report on the existence of caffeoylquinic acid in propolis. They isolated and identified caffeoylquinic acids from the water soluble portion of Brazilian propolis, whilst also showing that these compounds had the ability to stimulate macrophage mobility and spreading. This enhancement of macrophage activity was said to partly explain the immunomodulatory effects of propolis, as macrophages are known to be the first line of defence when tissues are affected by infection or injury. Additionally, Midorikawa *et al.* (2001) stated that caffeoylquinic acids in water and methanol extracts of Brazilian propolis had hepatoprotective and anti-oxidative properties, which were more profound in samples with higher caffeoylquinic acid concentrations. Caffeoylquinic acids have also been shown to inhibit lipid peroxidation in the liver and brain of rats, when these organs were subjected to oxidative stress (Kimura *et al.*, 1984; Nakajima *et al.*, 2007). This compound thus has potential to promote animal health and production and could thus be a valuable component in future feed additive studies. The chemical structure and molecular formula for caffeoylquinic acid can be seen in Figure 2.2, as obtained from the PubChem Substance and Compound Database using the chemical structure identifier, CID: 12310830 (National Center for Biotechnology Information, 2017).

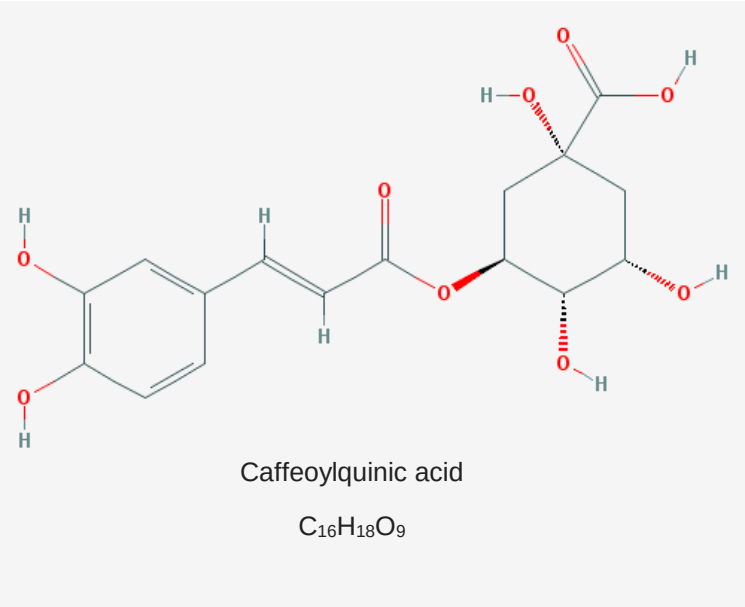

Figure 2.2 The chemical structure and molecular formula for caffeoylquinic acid according to the PubChem Substance and Compound Database, CID: 12310830 (National Center for Biotechnology Information, 2017)

2.5. Propolis as an AGP alternative in poultry nutrition

The pharmacological and nutraceutical benefits of propolis have been widely explored in numerous fields of medicine as a key resource for the treatment and prevention of oral and systemic diseases (Freires *et al.*, 2016). Alongside the extensive research into the benefits for human health, propolis has also been investigated as a diet supplement in domesticated poultry species (Mahmoud *et al.*, 2016). Despite the fact that propolis types vary greatly with location, reviews and research have been published giving a general idea of the effect on growth, carcass quality, immune status and organoleptic attributes in poultry; with the propolis type, origin or at least dietary concentrations as reference (Mahmoud *et al.*, 2016). Due to the lack of publications pinpointing and defining exact physiological impacts of propolis bio-active ingredients in the poultry sector, a more general overview of the reported propolis effects are discussed in this review. Table 2.1 gives a summary of past research that investigated the effects of different types and concentrations of propolis on different parameters when included in broiler chicken feeds.

2.5.1. Growth performance and efficiency

When considering the information in Table 2.1, it was seen that there was great variation in propolis types, origin and concentrations that have been tested in published literature. Despite these differences, the effect on growth and productivity is more prominently regarded as positive. Propolis extracts are known to have strong antibacterial actions, with the presence of micronutrients contributing positively to both broiler health and metabolism (Hosseini *et al.*, 2016). Corticosterone is a hormone known to cause protein catabolism and when birds are exposed to a stressor such as heat stress, production of this hormone has been shown to increase to levels where evidence of growth retardation was observed (Hayashi *et al.*, 1994; Quinteiro-Filho *et al.*, 2010). Propolis contains many active ingredients which are known for their potent antioxidant capabilities; and antioxidants have been shown to increase nutrient utilization, as well as reduce the production of corticosterone in birds, ultimately supporting growth under stressful conditions (Sahin *et al.*, 2003). This was demonstrated in a study by Chegini *et al.* (2017), who reported decreased corticosterone levels in propolis fed broilers, which was accompanied by improved production and reduced stress indicator measurements, when birds were exposed to heat stress and overcrowding.

Zafarnejad *et al.* (2017), Attia *et al.* (2014), and Shalmany and Shivazad (2006) reported that body weight gain (BWG) and FCRs were significantly improved with the addition of propolis in broiler diets. When broiler chickens were subjected to heat stress, Hosseini *et al.* (2016) and Seven *et al.* (2008) showed that the inclusion of propolis in the feed significantly increased BWG and feed intake (FI), while FCR was seen to be statistically similar to the unsupplemented control.

On the other hand, a study by Gheisari *et al.* (2017) showed that supplementation with propolis in broiler diets had no significant effect on BWG, FI or FCR in comparison to the control. Similar results were obtained by Daneshmand *et al.* (2015), Shalmany and Shivazad (2006), and Açikgöz *et al.* (2005) when propolis was included at lower concentrations in broiler diets. This indicated that these specific types of propolis may need to be included at higher concentrations so as to bring about significant results.

Table 2.1 A summary of past research that investigated the effects of different types and concentrations of propolis on different parameters in broiler chicken feeds

Broiler breed	Propolis origin	Propolis type	Propolis concentrations tested (mg/kg)	Production parameters*	Organ and immune parameters*	Carcass characteristics*	Organoleptic attributes*	References
Arbor Acres Broiler Chickens	Hangzhou Kangli Health Products Co. Ltd, China	Propolis	300	↑	↑	↑		(Attia *et al.*, 2014, 2017)
Ross 308 Broiler Chickens	Iran	Ethanol Extract	50, 100, 150, 200, 250	↑ (200, 250); = (50, 100, 150)				(Shalmany & Shivazad, 2006)
Ross 308 Broiler Chickens	Iran	Propolis Extract Powder	500, 1500, 2000	↑	↑	↑		(Shaddel-Tili *et al.*, 2017)
Ross 308 Broiler Chickens	Poland	Propolis Extract Powder	250			=		(Kleczek *et al.*, 2012)
Ross 308 Broiler Chickens	Brazil	Chemically standardised Propolis	10, 50	=		=		(Kleczek *et al.*, 2014)
Broiler Chickens (Male)	Brazil	Propolis Extraction Residue	10000, 20000, 30000, 40000	=	=			(Eyng *et al.*, 2015, 2017)
Ross 308 Broiler Chickens (Male)	Pine Tree Propolis, Turkey	Raw Propolis Powder	500, 2000, 4000	= (500, 2000); ↓ (4000)				(Açikgöz *et al.*, 2005)
Ross 308 Broiler Chickens (Male)	Iran	Ethanol Extract	200	=	↑	=		(Daneshmand *et al.*, 2015)
Ross 308 Broiler Chickens (Male)	Karaj, Alborz, Iran	Ethanol Extract	4000	↑	↑			(Chegini *et al.*, 2017)
Ross 308 Broiler Chickens (Male)	-	Propolis	600, 700, 800, 900	↑	↑	↑		(Zafarnejad *et al.*, 2017)
Ross 308 Broiler Chickens	-	Ethanol Extract	50, 100, 200, 300	=	=	=		(Gheisari *et al.*, 2017)
Ross 308 Broiler Chickens	-	Propolis Extract	500, 600			=		(Haščík *et al.*, 2015)
Ross 308 Broiler Chickens	-	Ethanol Extract	200, 300, 400			=		(Šulcerová *et al.*, 2011)
Ross 308 Broiler Chickens	-	Ethanol Extract	200, 300, 400				↑	(Haščík *et al.*, 2011)
Ross 308 Broiler Chickens (Heat Stress)	Eastern Anatolia	Ethanol Extract	500, 1000, 3000	↑		↑		(Seven *et al.*, 2008)
Ross 308 Broiler Chickens (Male) (Heat Stressed)	-	Ethanol Extract	3000	↑	↑			(Hosseini *et al.*, 2016)

↑ Improves; ↓ Worsens; = the same effect; * The effect was compared to a negative control

One study pointed out that if included at too high of a concentration, propolis can have an adverse effect on broiler production parameters. This was shown by Açikgöz *et al.* (2005) who, after obtaining no significant effects on growth parameters at 500 and 2000 mg/kg feed, doubled the concentration to 4000 mg/kg feed for a second broiler growth trial. The effect was a significant decrease in body weight and feed intake, although the FCR did not significantly differ from the control. This particular type of propolis, especially in raw form, was said to have a bitter taste and stringent odour which, when included at high concentrations, negatively affected the palatability of the feed (Açikgöz *et al.*, 2005). It is for this reason that propolis is usually treated with a solvent to obtain the more commonly used propolis ethanol extract; the raw material is seldom suitable for direct inclusion in an end product (Krell, 1996).

2.5.2. Organ parameters and immune response

The relative weight of the spleen and bursa of Fabricius is often used as a measure to predict the immune status of broiler chickens (Abdel-Fattah *et al.*, 2008). The bursa is responsible for B-cell development, while one of the spleens major functions is specific immune response. Lymphoid organs thus play a key role in defending the body against harmful micro-organisms (Cooper *et al.*, 1966). Differences in relative weights may therefore be related to changes in the efficiency or functionality of lymphoid organs (Cooper *et al.*, 1966). It was found that through years of artificial selection for increased body weight, the modern day commercial broiler strain exhibited much smaller relative sizes of both primary and secondary lymphoid organs (Cheema *et al.*, 2003). This outcome was referred to as the “resource allocation theory” (Rauw *et al.*, 1998) and affected the bird’s ability to maintain their health and immuno-competence (Cheema *et al.*, 2003). Relative lymphoid organ weights should not be used solely to predict immune response, as the lowest weight may not necessarily be linked to decreased lymphoid cell production. It is recommended that these weights be correlated with another measure of immune response before coming to any final conclusions (Kabir *et al.*, 2004).

Fan *et al.* (2013) showed that a propolis-polysaccharide treatment combination increased antibody titer and lymphocyte proliferation in high and medium dosed broiler groups. This effect was accompanied by significantly larger lymphoid organs. Similar results were demonstrated by Attia *et al.* (2017) after they included propolis at 300 mg/kg in broiler diets. These results indicated that immune function was improved in supplemented birds, as their ability to resist the effect of immunosuppression on lymphoid organ development was heightened. Similarly, Zafarnejad *et al.* (2017) and Chegini *et al.* (2017) reported a significant increase in spleen and bursa relative weights, with Zafarnejad *et al.* (2017) also demonstrating an increased antibody response in propolis fed broilers (900 mg/kg feed). These results too confirmed that propolis had the ability to modulate humoral immunity in broilers.

Literature further indicated that propolis has improved immune response in broilers exposed to heat stress (Hosseini *et al.*, 2016). Heat stress is a well-known stressor in the poultry industry and has been shown to induce atrophy in lymphoid organs, subsequently reducing bursa, spleen and thymus relative weights (Quinteiro-Filho *et al.*, 2010). Hosseini *et al.* (2016) used biomarkers and other heat stress tests to measure the effect of propolis and heat stress on broiler chickens. The tests and relative spleen and bursa weights confirmed that propolis, as a feed additive, had the ability to overcome the damaging effects of this stressor.

2.5.3. Carcass characteristics and organoleptic attributes

Faster growing birds have heavier body weights at slaughter, and typically then also have higher carcass weights and dressing percentages. It was shown that when propolis enhanced body weight gain in broilers, a significant increase in carcass weight and dressing percentage followed (Attia *et al.*, 2014; Zafarnejad *et al.*, 2017). Similarly, where differences in growth rates did not occur, carcass weights and dressing percentages too did not differ from the unsupplemented control (Daneshmand *et al.*, 2015; Gheisari *et al.*, 2017). In a study where propolis was investigated in heat stressed broilers, significant increases were seen in carcass yield and relative breast meat weights at an inclusion of 1000 mg/kg feed of propolis extract. The stunted growth of the control group in comparison, indicated that propolis can potentially be used to combat negative effects of heat stress in broilers. Relative leg, wing, neck and back weights, did not differ significantly between any of the tested groups (Seven *et al.*, 2008).

Meat quality is highly dependent on the rate of pH decline and the ultimate pH of the muscle after slaughter (Sales & Mellett, 1996). Glycolytic enzymes break down glycogen to glucose after death, which in the absence of oxygen forms lactic acid through the process of glycolysis. Lactic acid build up reduces the muscle pH, and this decline aids in the conversion of muscle to meat (Murray, 1995). Ultimate pH is thus largely dependent on the pre-slaughter glycogen reserves of the muscle (Mellor *et al.*, 1958). Muscle pH and meat colour are highly correlated. The rate of muscle pH decline can also be associated with the meat's drip loss, cooking loss and shelf-life (Allen *et al.*, 1998; Šulcerová *et al.*, 2011). A low muscle pH is typically associated with reduced water holding capacity and visibly lighter coloured meat; while a higher muscle pH creates a more favourable environment for bacterial growth which in turn reduces shelf-life (Allen *et al.*, 1998). Undesirable effects that are related to oxidative reactions in food include unpleasant flavours and odours, colour loss and reduction in nutritional value (Viuda-Martos *et al.*, 2008). Due to its naturally high antioxidant potential, propolis has the ability to promote oxidative stability in meat through mechanisms such as hydrogen donation, free radical scavenging and metallic chelation (van Acker *et al.*, 1996). Studies which have tested post mortem pH and colour in broiler meat showed no significant differences between propolis-fed birds and the control, however, significant correlations between pH and colour were noted (Šulcerová *et al.*, 2011; Haščík *et al.*, 2015).

The sensory profile of meat is another major determining factor of meat quality. Sensory analysis is one of the oldest forms of quality control and is still actively used today to understand and predict consumer preference, which in turn promotes competitiveness in the market place (Gridgeman, 1967; Haščík *et al.*, 2011). Attributes such as aroma, texture and flavour all influence consumer satisfaction and the likeliness of repurchase (Fletcher, 2002; Pieterse *et al.*, 2014). Studies have shown that the use of certain phytogenic additives in broiler feeds could somewhat enhance the sensory profile of meat. Some phytogenic examples that had this effect in broiler meat include: propolis (Haščík *et al.*, 2011), garlic and oregano essential oils (Kirkpinar *et al.*, 2014), garlic bulb and husk powder (Kim *et al.*, 2009), and dried milfoil and St. John's-wort herbage (Fritz *et al.*, 1993). Many of the active ingredients in propolis and these phytogenics are known for their antioxidant capabilities and oxygen-derived free radical scavenging capabilities (Pascual *et al.*, 1994; Viuda-Martos *et al.*, 2008). Dietary supplementation with these compounds can therefore improve texture, flavour and other sensory attributes, by potentially delaying post mortem oxidative deterioration of meat and lipids (particularly

polyunsaturated fatty acids), which is a process responsible for the development of rancid odours and flavours in meat (Wood & Enser, 1997; Antony *et al.*, 2006; Viuda-Martos *et al.*, 2008).

Negative effects that have arisen due to broiler selection programs selecting for increased bodyweight and growth rates, come to include higher occurrences of leg problems; more specifically bone abnormalities and deformities (Leterrier & Nys, 1992; Paxton *et al.*, 2013). The skeletal system is responsible for body movement and posture; protection of organs and tissues; and it serves as the primary store for the mineral compounds calcium, magnesium and phosphorus. It was seen by Stójko *et al.* as far back as 1978, that an ethanol extract of propolis had the ability to accelerate bone tissue regeneration. More recently, Ang *et al.* (2009) demonstrated that caffeic acid phenethyl ester (CAPE), a bioactive component of propolis, had the potential to treat bone lytic diseases in humans. Propolis has also been found to benefit skeletal components by significantly improving the utilization and availability of dietary calcium and phosphorous (Haro *et al.*, 2000).

Research on the effects of propolis on skeletal parameters in poultry, however, is still limited and lacking. One study reported that after analysing the biochemical properties of tibias in broiler chickens, it was found that propolis-fed diets did not lead to any significant differences in the shear strength values or physical properties of these bones. This particular study did however show that where the concentrations of phosphorous in ashed tibias did not differ, the calcium concentrations were significantly lower for propolis-fed broilers (Kleczek *et al.*, 2012). Further research and measurement techniques are needed to investigate these effects more thoroughly, while keeping in mind that numerous factors are known to affect bone strength in poultry, including age, sex, nutrition, genetic factors, type of production system and management conditions.

2.6. Conclusion

In conclusion, with the practice of banning AGP-use becoming more widely adopted, alternative solutions are required for the replacement of these additives with alternatives that can bring about similar effects, i.e. by efficiently improving the health and production of livestock simultaneously. In South Africa especially, where the risk of AMR-related deaths is high, due to the overuse and misuse of antimicrobials, accompanied by poverty, infectious diseases and the lack of awareness; the application of natural alternatives to AGPs is vital and necessary to reduce the chances of AMR transmission and ultimately to relieve this negative pressure on public health. Natural growth promoters or phytogenics; such as the bee product, propolis; have considerable potential for AGP replacement. The many bio-active ingredients found to exist in propolis (for example: caffeoylquinic acid and the bioflavonoid, prodelphinidin), have numerous biological activities (i.e. antimicrobial, antioxidant, antiviral, etc.) which have been shown to significantly improve animal health and production. With regards to the broiler industry, however, much of the published research is lacking proper classification of the propolis types used, which makes adequate comparisons between articles challenging, and in some cases impossible. This study will thus aid in demonstrating the effects of specific bio-active ingredients that have been identified and isolated from propolis, on broiler health and production; so as to give better insight into the use and potential of propolis and its components in broiler nutrition in the future.

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Chapter 3

The effect of a phytogenic feed additive on the production parameters of broiler chickens

Abstract

A 35 day trial was conducted to investigate the effect of the phytogenic feed additive, VivoCare®, on the production parameters of mixed sex, Cobb 500 broiler chickens. The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd, after studying signal molecules and gene expression in different types of propolis, and it contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components. The trial consisted of five experimental diets, each replicated six times; and which included a positive control, a negative control, and three test diets with inclusion levels of 500, 600 and 800 mg of VivoCare® per kg of feed. There were no significant differences between any of the diets for weekly live weights, overall average live weight gain, overall average feed intake, feed conversion ratio (FCR), liveability, average daily gain (ADG), protein efficiency ratio (PER) or European production efficiency factor (EPEF). Average weekly live weight gain and feed intake did not differ significantly for all of the weeks except for the week of Day 15 - 21, where birds fed diet P600 had the highest average live weight gain and feed intake, while the negative control had the lowest. The results indicated that all of the diets performed equally well in terms of overall growth performance. The lack in differences may have been due to the trial being conducted under optimal environmental conditions and it was recommended that further research be conducted with VivoCare® in a trial where an intentional stressor is applied, as this may promote more noticeable results.

Keywords: AGP, caffeoylquinic acid, EPEF, FCR, feed intake, live weight, PER, prodelphinidin, propolis, weight gain

3.1. Introduction

The discovery of antimicrobials is said to be one of the most significant accomplishments in modern medicine. With regards to animals for food production, antimicrobials have been used to cure and prevent disease, but also to promote growth as antimicrobial growth promoters (AGPs) (Laxminarayan *et al.*, 2015). When it was discovered that the use of antimicrobials could promote health and animal productivity simultaneously, the inclusion of AGPs as feed additives became very popular (Laxminarayan *et al.*, 2015). It was pointed out that in optimal environmental conditions, the addition of AGPs to animal feeds had no growth-promoting effect (Coates *et al.*, 1955, 1963). As many livestock production facilities were not always adequately pathogen- and stress-free, especially in less developed countries, farmers became familiar with the use of AGPs to guarantee optimal health and production, sometimes relying on the ability of these additives to act as a substitute for hygiene-management practices (Key & Mcbride, 2014; Laxminarayan *et al.*, 2015).

A major concern that arose alongside the extensive use of AGPs, was the impeding threat of antimicrobial resistance (AMR) (Swann *et al.*, 1969). The use and over-use of antimicrobials in livestock, for whatever

purpose, can lead to a build-up of antimicrobial-resistant micro-organisms in the animal and subsequently in the animal products. These resistant microbial residues can then potentially be transmitted to humans when these products are consumed (Smith *et al.*, 2002; World Health Organisation, 2015). Antimicrobial resistant micro-organisms threaten the effectiveness of human drugs if these microbes or genes become incorporated into the microflora populations colonizing the gastrointestinal tract in humans, and this in turn poses a serious threat to public health (Smith *et al.*, 2002). In 1986, Sweden became the first nation to ban the use of antimicrobials as AGPs in animal feeds, and by 2006 the European Union had banned this practice altogether (Wierup, 2001; European Commission, 2005).

In the 21st century, efforts have turned to finding viable options for the replacement of AGPs. Examples of feed additives that have been tested for this purpose in broiler nutrition, come to include organic acids, probiotics, prebiotics and plant extracts (also known as phytogenics) (Grashorn, 2010). The use of phytogenics as feed additives still requires much research with regards to their modes of action, application, and potential interactions with other additives in animal nutrition; as this category of feed additive is still relatively new. Phytogenic feed additives (PFAs) have been classified as sensory or flavouring compounds, which predominantly consist of plant extracts (i.e. flavonoids, essential oils, and oleoresins) and their bio-active components from which their numerous beneficial properties are derived (Murugesan *et al.*, 2015).

Propolis, a prime example of a phytogenic, is a natural resin produced and used by honey bees from collected plant exudates to protect the hive, their larvae and themselves from harmful microbes (Banskota *et al.*, 2001). Due to its extensive range of bio-active ingredients, propolis has received much attention for its numerous beneficial biological and pharmacological properties (Farooqui & Farooqui, 2012). Various studies have demonstrated the beneficial effects of propolis against many types of micro-organisms, including viruses (Gekker *et al.*, 2005), bacteria (Dziedzic *et al.*, 2013), fungi (Murad *et al.*, 2002), and moulds (Miyataka *et al.*, 1997); while also showing that propolis had the ability to exert anti-inflammatory, immunomodulatory, antiparasitic, antioxidant and other protective functions (Farooqui & Farooqui, 2012; Attia *et al.*, 2017).

Studies that have looked at the effects of propolis on production parameters in broiler chickens have yielded inconsistent results. Some studies showed that growth performance was improved with the supplementation of propolis-based products in the feed (Shalmany & Shivazad, 2006; Attia *et al.*, 2014; Zafarnejad *et al.*, 2017); while other studies indicated that supplementation, especially at lower concentrations, had no effect on growth performance (Açikgöz *et al.*, 2005; Shalmany & Shivazad, 2006; Daneshmand *et al.*, 2015; Eyng *et al.*, 2017; Gheisari *et al.*, 2017). Research has also shown that when broilers were subjected to heat stress, birds that were fed the propolis-supplemented diets grew significantly better than the negative control. This indicated that propolis-based products may have the ability to combat the negative effects of heat stress (Seven *et al.*, 2008; Hosseini *et al.*, 2016; Chegini *et al.*, 2017). One study, by Açikgöz *et al.* (2005) demonstrated that if used in its raw form at too high a concentration, propolis could significantly reduce growth performance due to possible negative effects on palatability.

In this study, three different concentrations of the phytogenic feed additive, VivoCare®, were supplemented in the diets of broiler chickens. This unique product was produced by Beonics Feed Supplements (Pty) Ltd, after studying certain signal molecules and gene expression in different types of propolis. The major bio-active

ingredients of this product include caffeoylquinic acid and prodelphinidin bioflavonoids; both of which are known for their antimicrobial and antioxidant capabilities, among other beneficial biological effects (Midorikawa *et al.*, 2001; Rodríguez-Pérez *et al.*, 2016; Yang *et al.*, 2016). The results were compared to both a positive and negative control to determine the effects on the following production parameters: average live weight gain, average feed intake, feed conversion ratio (FCR), liveability, average daily gain (ADG), protein efficiency ratio (PER) and European production efficiency factor (EPEF).

3.2. Materials and Methods

3.2.1. Experimental birds, layout and housing system

Prior to the commencement of the trial, ethical clearance was obtained from the Animal Ethics Committee of Stellenbosch University; reference number SU-ACUD16-00018. A total of 330 day-old, mixed-sex, Cobb 500 broiler chicks were obtained from a commercial hatchery (County Fair, Hatchery 5, Klapmuts, Western Cape, South Africa). Chicks had been vaccinated against Infective Bronchitis, as well as Newcastle Disease. Chicks were placed in a temperature controlled house with artificial lighting at the Poultry Section of the Mariendahl Experimental Farm (Stellenbosch University). The temperature, ventilation and lighting of the house was set and controlled according to the COBB Broiler Management Guide (2013).

The trial consisted of five treatments replicated six times and was set out in a completely randomised manner. There were thus a total of 30 wire cages each containing 11 chicks that were allocated randomly. The cages were 1.86 m by 0.61 m in size, elevated 1.07 m off the floor, and each contained two nipple drinkers and a tube feeder. Chicks received feed and water *ad libitum* throughout the trial. The trial ran for a period of 35 days.

3.2.2. Experimental treatments and diet formulations

During the trial the chicks were fed a three phase mash diet consisting of a starter, grower and finisher. These diets were formulated according Cobb 500 requirements (Cobb 500 Broiler Performance and Nutrition Supplement, Cobb-Vantress Inc., Siloam Springs, AR). Diets were allocated so that each chick received 900 g of starter (consumed during ± 18 days), 1200 g grower (consumed during ± 7 days), and then finisher up until the day of slaughter (consumed during ± 10 days). As the test additives were non-nutritive, the same feed formulation was used for each treatment diet with the addition of specific feed additives included at a rate of up to 800 mg/kg of feed. The ingredient and nutrient composition of the starter, grower and finisher diets can be seen in Table 3.1. The feeds were mixed at Mariendahl Experimental Farm (Stellenbosch). The inclusion rates for each additive was based on the manufacturer's recommendations. The treatments consisted of a negative control containing no AGP (NEG); a positive control containing a commercial AGP (POS), namely Zinc Bacitracin included at 150 mg/kg of feed; and three diets which each contained a different concentration of the phytogenic feed additive, VivoCare®, provided by Beonics Feed Supplements (Pty) Ltd. These three inclusion levels were 500, 600 and 800 mg of VivoCare® per kg of feed and are further referred to as P500, P600 and P800, respectively. A description of each experimental treatment diet can be seen in Table 3.2. Bentonite was included as an inert filler when test additives had inclusions of less than 800 mg/kg of feed.

Table 3.1 Ingredients and calculated nutrient composition of broiler starter, grower and finisher diets used in the trial

	Starter	Grower	Finisher
Ingredients (g/kg)			
Maize meal	450.90	622.90	539.43
Full fat soybean meal	115.28	152.30	274.73
Soya bean meal (47% CP[1])	277.79	175.80	100.00
Fish meal	53.62	-	-
L-lysine HCl	1.78	2.50	1.67
DL methionine	3.97	3.00	3.25
L-threonine	1.14	0.60	0.95
Vitamin and mineral premix	2.00	2.00	2.00
Limestone	14.47	12.20	14.37
Salt	1.23	3.60	2.61
Monocalcium phosphate	12.19	14.60	13.43
Sodium bicarbonate	1.63	-	1.48
Sunflower oil	61.99	10.00	45.58
Calculated nutritional value (g/kg)			
Dry matter	887.03	880.99	886.34
Moisture	112.97	119.01	113.66
AME[2] (MJ/kg)	12.65	13.00	13.40
Crude protein	167.19	205.63	166.25
Crude fat	121.92	67.33	120.02
Crude fibre	52.44	38.29	42.13
Ash	58.16	46.99	54.80

[1]Crude Protein
[2]Apparent metabolizable energy

Table 3.2 A description of the five experimental treatments used throughout the trial

Treatment	Inclusion	Description
P500	500 mg/kg inclusion	Different inclusion levels of phytogenic product, VivoCare® (Beonics Feed Supplements (Pty) Ltd)
P600	600 mg/kg inclusion	
P800	800 mg/kg inclusion	
POS	150 mg/kg inclusion	Positive Control (AGP[1] - Zinc Bacitracin)
NEG	No AGP[1]	Negative control

[1]Antimicrobial growth promoter: Zinc Bacitracin

3.2.3. Production parameter data collection

Body weight was measured per cage on arrival, as well as on a weekly basis up until the day of slaughter at 35 days of age, using an LBK ADAM® scale (Adam Equipment Co Ltd, Kempton Park, Johannesburg). Individual weights were calculated based on the average of each cage, after correcting for mortalities. Weekly feed intake was calculated according to the total feed consumed per week per cage up until the day of slaughter. Thereafter, the average feed intake per bird per week was determined after correcting for mortalities. Mortalities were recorded twice daily, and dead bird weights and the feed remaining at the time of death was documented (LBK ADAM® scale). From this data it was possible to determine live weight gain, ADG, FCR (Equation 3.1), liveability (Equation 3.2), EPEF (Equation 3.3) and PER (Equation 3.4).The formulae for these equations are as follows:

Equation 3.1: $$\text{FCR} = \frac{\text{Cumulative feed intake per chick (g)}}{\text{Average live weight gain (g)}}$$

Equation 3.2: $$\text{Liveability} = \frac{\text{Number of birds that survived until slaughter}}{\text{Total number of birds at start}} \times 100$$

Equation 3.3: $$\text{EPEF} = \frac{\text{Liveability}(\%) \times \text{Live weight gain (kg)}}{\text{Age (days)} \times \text{FCR}} \times 100$$

Equation 3.4: $$\text{PER} = \frac{\text{Live weight gain (g)}}{\text{Crude protein intake (g)}}$$

3.2.4. Statistical analysis

The study represented that of a completely randomised design with treatments as the main effect. All statistical procedures were performed using SAS® statistical software (SAS Enterprise Guide 2014, Version 7.1, SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was conducted to test for the non-normality of residuals (Shapiro & Wilk, 1965), and Bartlett and Levene's tests were performed to test for heteroscedasticity (Bartlett, 1937; Levene, 1960). With the latter assumptions satisfied, Analysis of Variance (ANOVA) was performed on the cage averages by means of the general linear model (GLM) procedure. Differences were said to be significant when $P < 0.05$. If significant differences existed, multiple comparison *post-hoc* tests were performed using Bonferroni (Dunn) t Tests. When calculating the ADG, a simple linear regression model of weight over

time was fitted. The gradient of this regression function was subsequently used to represent ADG. ANOVA and *post-hoc* tests were then performed on these gradient values so as to compare ADG between treatments.

3.3. Results and Discussion

3.3.1. Live weight and live weight gain

The experimental diets included both a positive and negative control. The negative control was AGP free and results from this diet were unofficially regarded as the "worst-case scenario"; whilst the positive control contained a commercially used and available AGP (Zinc Bacitracin), the so-called "best-case scenario". The study was conducted with the assumption that in order for VivoCare® to have any potential viability in a commercial setting, it had to out-perform the negative control, unless the positive and negative control were statistically similar. Comparisons were largely based on these assumptions throughout the study.

The average live weights and live weight gains at weekly intervals for broilers grown from hatch to 35 days for the five experimental diets (P500, P600, P800, POS and NEG) are provided in Table 3.3 and Table 3.4, respectively. No differences (P >0.05) in average live weights existed between any of the treatment groups at each of the five weekly measuring points. In terms of weekly average live weight gain, a difference (P <0.05) was only seen for the time period of Day 15 - 21, where diet P600 was significantly higher than the negative control. The periods of Day 0 - 7, 8 - 14, 22 - 28, 29 - 35, and the overall average live weight gain (Day 0 - 35) did not indicate any differences (P >0.05) between the treatment groups. The spike of the average live weight gain for P600 versus the negative control for the period of Day 15 - 21 was thus not major enough to cause a significant overall weight gain difference.

The fact that AGPs may not bring about growth-promoting effects in pathogen-free animals, may possibly be the reason for the statistical similarities between the treatment groups (Coates *et al.*, 1955, 1963). As birds were not subjected to any deliberate stressors, and the negative control performed as well as the positive control and the other three test groups for both average live weight and live weight gained, it could be assumed that the birds in this study were raised in a reasonably stress- and pathogen-free environment. Similar results were seen in a study by Gheisari *et al.* (2017) where intentional stressors too were absent. It was seen that no significant differences occurred in broiler average body weights at days 21 and 42 between the positive control, negative control and propolis ethanol extract treatments (50, 100, 200, 300 mg/kg). On the other hand, when Seven *et al.* (2008) tested the effect of a propolis ethanol extract on heat stressed birds, the positive control for each of the five weeks had a significantly higher live weight and live weight gain than the negative control; while the highest of the propolis extract concentrations (3000 mg/kg) performed at an intermediary level between that of the positive and negative control. When testing a new feed additive as a potential AGP replacer, it is thus necessary to see the effect on growth and production in all potential environments (stressor-free and stressor-induced) so as to decide on the most suitable form and concentration for future use.

Table 3.3 Average (± standard error) live weights (g) at weekly intervals for broilers grown from hatch to 35 days receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
P500	42.9 ± 0.60	165.6 ± 2.41	449.7 ± 6.21	897.3 ± 15.6	1485.3 ± 22.1	2213.0 ± 37.7
P600	44.3 ± 0.34	164.9 ± 1.92	454.1 ± 3.51	926.3 ± 10.2	1534.2 ± 12.3	2255.0 ± 21.7
P800	44.4 ± 0.52	160.4 ± 2.52	443.7 ± 5.61	909.4 ± 9.3	1513.0 ± 19.9	2215.4 ± 31.3
POS	44.3 ± 0.41	161.8 ± 1.61	445.9 ± 6.30	899.7 ± 9.1	1499.1 ± 17.4	2218.4 ± 31.2
NEG	43.3 ± 0.25	163.0 ± 1.37	440.8 ± 3.70	875.8 ± 10.6	1457.1 ± 17.1	2159.3 ± 26.5
***P* value**	0.072	0.355	0.426	0.054	0.061	0.302

[a,b] Means within columns with different superscripts differ significantly (P <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 3.4 Average (± standard error) weekly and overall live weight gains (g) for broilers grown from hatch to 35 days receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Day 0 - 7	Day 8 - 14	Day15 - 21	Day 22 - 28	Day 29 - 35	Day 0 - 35
P500	122.7 ± 2.17	284.1 ± 4.07	447.6^{ab} ± 10.67	588.0 ± 7.71	727.7 ± 19.37	2170 ± 37.5
P600	120.6 ± 1.73	289.2 ± 2.33	472.2^{a} ± 7.08	607.9 ± 3.12	720.8 ± 15.05	2211 ± 21.5
P800	116.0 ± 2.56	283.4 ± 3.43	465.6^{ab} ± 7.95	603.7 ± 11.1	702.4 ± 12.03	2171 ± 31.6
POS	117.5 ± 1.91	284.1 ± 5.00	453.8^{ab} ± 4.11	599.4 ± 12.6	719.4 ± 15.47	2174 ± 31.2
NEG	119.7 ± 1.26	277.7 ± 2.73	435.1^{b} ± 7.47	581.2 ± 7.28	702.2 ± 16.15	2116 ± 26.4
***P* value**	0.165	0.315	**0.019**	0.226	0.709	0.312

[a,b] Means within columns with different superscripts differ significantly (P <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

3.3.2. Feed Intake

The average weekly and overall feed intake for broilers grown from hatch to 35 days for the five experimental diets (P500, P600, P800, POS and NEG) is provided in Table 3.5. There were no differences (P >0.05) between treatment groups for the time periods of Day 0 - 7, 8 - 14, 29 - 35 and the overall average feed intake (Day 0 - 35). There was, however, a difference (P <0.05) at the time period of Day 15 - 21, where diet P600 had a significantly higher feed intake compared to the negative control. The percentage increase in feed intake during this week for diet P600 birds was 84.3%, while the negative control only had a 74.5% increase. For the period of Day 22 - 28 the ANOVA had indicated that differences between the groups were present (P <0.05), however, when *post-hoc* comparison tests (Bonferroni t Tests) were performed, differences became

insignificant between the treatment groups and it was decided that the results for this particular week would be accepted as statistically similar.

It was believed that the previously noted difference in average weight gain between treatment P600 and the negative control for Day 15 - 21 (Table 3.4) was as a result of the change in feed intake for this particular week. It is generally accepted that the higher the average feed intake, the greater the average weight gain; and *vice versa* (Teeter & Smith, 1985). The cause for this arbitrary change in feed intake could have potentially been related to the transition from the starter to the grower diet during this week. It is possible that the transition led to an amino acid imbalance in the control birds; or that an imbalance may have occurred due to the change in broiler requirements during this time period (e.g. for feather growth, immune response, etc.). It is well known that amino acid imbalances suppress feed intake and growth, however, it is recommended that the results from this study be further investigated as this change may have occurred due to a wide range of contributing factors.

Feed additives can sometimes have a negative effect on the quality and palatability of a feed, depending on the form and concentration in which they are included. Poor feed quality or palatability may result in a decrease in feed intake and this in turn may stunt growth. Açikgöz *et al.* (2005) showed that when a high concentration (4000 mg/kg) of raw propolis in powdered form was added to broiler diets, feed intake and body weight gain were significantly lower than the control. This form of propolis was said to have a stringent odour and bitter taste (Açikgöz *et al.*, 2005). Propolis is thus more commonly used in an ethanolic extract form for direct inclusion in end products (Krell, 1996). As there was no overall difference in average feed intake between any of the treatment groups, it was assumed that the form and concentrations of the additives used in this trial, had no negative effects on the feed quality and palatability.

The lack of significant differences in feed intake also supported the theory that the birds were raised in a reasonably stress- and pathogen-free environment. Birds who are subjected to some form of stressor often refuse to eat, which reduces feed intake and subsequent weight gain. This effect was seen in trials where ethanolic extracts of propolis was fed to heat stressed birds; whereby the negative control had significantly lower overall feed intake in comparison to the positive control and propolis-based diets (Seven *et al.*, 2008; Hosseini *et al.*, 2016). This also showed that propolis had the potential to reduce the negative effects of heat stress on broilers.

Table 3.5 Average (± standard error) weekly and overall feed intake (g) for broilers grown from hatch to 35 days receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Day 0 - 7	Day 8 - 14	Day15 - 21	Day 22 - 28	Day 29 - 35	Day 0 - 35
P500	126.1 ± 2.61	360.1 ± 3.46	643.6^{ab} ± 9.02	989.9 ± 10.9	1301 ± 22.0	3420 ± 40.2
P600	126.1 ± 1.00	361.4 ± 2.09	665.9^{a} ± 4.76	1033.9 ± 5.58	1333 ± 18.6	3520 ± 24.5
P800	126.4 ± 5.94	353.6 ± 3.98	641.8^{ab} ± 6.94	1002.5 ± 13.9	1289 ± 13.9	3413 ± 38.5
POS	124.3 ± 1.61	358.7 ± 2.48	643.1^{ab} ± 8.12	1010.1 ± 15.4	1319 ± 11.3	3455 ± 33.7
NEG	126.1 ± 3.12	359.9 ± 5.15	628.2^{b} ± 5.52	982.0 ± 12.1	1284 ± 11.0	3380 ± 28.8
***P* value**	0.991	0.596	**0.017**	0.049[1]	0.188	0.068

^{a,b} Means within columns with different superscripts differ significantly (P <0.05); [1]Accepted as statistically insignificant after performing *post-hoc* comparison tests; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

3.3.3. Feed conversion ratio

The average cumulative FCR for broilers grown from hatch to 35 days can be seen in Table 3.6. No differences (P >0.05) existed between any of the experimental diets (P500, P600, P800, POS and NEG) at any of the cumulative time periods.

Similar results, where significant differences in FCR did not occur, have been seen in both stressor-free and heat-stress-induced environments where propolis ethanol extracts were tested for their potential to replace AGPs (Açikgöz *et al.*, 2005; Seven *et al.*, 2008; Daneshmand *et al.*, 2015; Hosseini *et al.*, 2016). On the other hand, it has also been reported that FCRs were significantly improved with the addition of ethanolic extracts of propolis in broiler diets (Shalmany & Shivazad, 2006; Attia *et al.*, 2017; Chegini *et al.*, 2017; Zafarnejad *et al.*, 2017).

Table 3.6 Average (± standard error) cumulative feed conversion ratios for broilers grown from hatch to 35 days receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Day 0 - 7	Day 0 - 14	Day 0 - 21	Day 0 - 28	Day 0 - 35
P500	1.03 ± 0.020	1.20 ± 0.012	1.32 ± 0.013	1.47 ± 0.009	1.58 ± 0.012
P600	1.05 ± 0.013	1.19 ± 0.010	1.31 ± 0.009	1.47 ± 0.006	1.59 ± 0.008
P800	1.09 ± 0.041	1.20 ± 0.017	1.30 ± 0.015	1.45 ± 0.014	1.57 ± 0.015
POS	1.06 ± 0.010	1.20 ± 0.016	1.32 ± 0.009	1.47 ± 0.005	1.59 ± 0.009
NEG	1.05 ± 0.022	1.22 ± 0.013	1.34 ± 0.011	1.48 ± 0.010	1.60 ± 0.014
***P* value**	0.488	0.559	0.155	0.136	0.534

^{a,b} Means within columns with different superscripts differ significantly (P <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

3.3.4. European production efficiency factor, liveability, average daily gain and protein efficiency ratio

The European production efficiency factor (EPEF), liveability (%), average daily gain (ADG) and protein efficiency ratio (PER) for broilers grown from hatch to 35 days can be seen in Table 3.7. No differences (P >0.05) existed between any of the five experimental diets (P500, P600, P800, POS and NEG) for EPEF, liveability, ADG or PER.

As indicated by Equation 3.3, EPEF is calculated using liveability (%), live weight gain, age and FCR of the birds at the end of the trial. The larger the EPEF value, the better the overall technical performance. The lack of significant differences in EPEF values was also found in a study by Kleczek *et al.* (2014) who was investigating the effect of propolis on broiler growth performance.

Liveability represents the percentage of birds that survived up until slaughter. Low mortality rates accompanied by no significant differences in liveability, indicated that birds were in good health which again supports the assumption that the birds in this trial were raised under optimal environmental conditions. Similar research by Gheisari *et al.* (2017) found that propolis ethanol extracts had no significant effects on mortality rates, while other studies showed that the inclusion of propolis, especially at higher concentrations, significantly reduced mortality rates (Shalmany & Shivazad, 2006; Zafarnejad *et al.*, 2017).

The PER is based on the amount of body weight gained divided by the amount of feed protein consumed during a particular testing period (Equation 3.4). The PER is most often used to evaluate the protein quality in a feed (Johnson & Parsons, 1997). Due to the fact that the test additives in this study were non-nutritive and the same feed formulations were thus used for all of the experimental diets, the protein quality and content amongst the treatment diets were assumed to be constant. Significant differences in PER were thus not expected between the treatment diets, especially since there were no overall differences (P >0.05) in body weight gain or feed intake (from which crude protein intake is derived).

Table 3.7 Average (± standard error) EPEF[1], liveability (%), ADG[2] and PER[3] for broilers grown from hatch to 35 days receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	EPEF[1]	Liveability (%)	ADG[2] (g)	PER[3]
P500	388.36 ± 9.43	98 ± 2	62.45 ± 0.36	2.66 ± 0.03
P600	380.65 ± 9.74	95 ± 3	63.08 ± 0.37	2.65 ± 0.02
P800	399.44 ± 8.88	100 ± 0	62.45 ± 0.36	2.66 ± 0.03
POS	374.40 ± 16.19	95 ± 3	62.30 ± 0.36	2.64 ± 0.02
NEG	380.77 ± 7.17	100 ± 0	61.97 ± 0.38	2.61 ± 0.02
***P* value**	0.537	0.195	0.370	0.590

[a,b] Means within columns with different superscripts differ significantly (P <0.05); [1]European production efficiency ratio; [2]Average daily gain; [3]Protein efficiency ratio; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

3.4. Conclusion

Overall, the results showed that the negative control, positive control and three VivoCare® test diets performed equally well in terms of growth performance. A possible reason for the lack in differences could have been due to the birds being raised in an optimal environment that was reasonably stress- and pathogen-free. The lack of differences in feed intake also confirmed that the form and concentration of the tested additives had no negative effects on feed quality and palatability. The temporary difference in feed intake and live weight gain that was noted for week 3 between diet P600 and the negative control, could have potentially been due to amino acid imbalances possibly caused by the transition from starter to grower diets and/or by the change in bird requirements during this time period. Further research should, however, be conducted as effects could potentially become more profound in a more challenging environment i.e. when an intentional stressor (i.e. heat or deliberate imbalances) is applied.

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Chapter 4

A toxicity study to determine the effect of a phytogenic feed additive on organ and intestinal parameters

Abstract

The purpose of this study was to investigate the effect of the phytogenic feed additive, VivoCare®, on the organ and intestinal parameters of mixed sex, Cobb 500 broiler chickens. The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd, and contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components, identified from propolis. In a primary trial, five experimental diets were tested, each with six replications. The experimental diets consisted of a positive control, a negative control, and three VivoCare® test diets (500, 600 and 800 mg of VivoCare® per kg of feed; P500, P600 and P800). A secondary trial investigated the effect of a double dosage of VivoCare® (1600 mg/kg) on the gizzard erosion score of broilers at 14 days of age. There were no significant differences in gizzard erosion scores for either of the trials. For the gizzard, heart, liver, spleen and bursa, significant differences were only observed for the spleen average weights and relative weights at day 35; whereby negative control birds had significantly larger spleen relative weights compared to the P800 group. No significant differences were observed for intestinal pH, while liver colour measurements indicated that the negative control had livers which were significantly more yellow in comparison to diet P600 at day 35. Overall, there was little evidence to suggest any major differences in immune status and gut health between the experimental groups. The negative control showed slightly more evidence of exposure to immunological stress, however, differences were not prominent and further research is recommended to support these results.

Keywords: caffeoylquinic acid, gizzard erosion, gut health, immune response, intestinal pH, liver colour, prodelphinidin, propolis, relative organ weights

4.1. Introduction

The gastrointestinal tract (GIT) is primarily responsible for nutrient digestion and absorption, however, it is also the largest immunological organ in the body. Anything that affects the health of the GIT will consequently influence the requirements and uptake of nutrients in this system, thereby affecting the animal's overall growth and performance (Choct, 2009). Research has shown that antimicrobial growth promoters (AGPs) have the ability to maintain and enhance gut health through their direct effect on the intestinal microflora; by reducing opportunistic pathogens and subclinical infections; and by reducing gut thickness and size (Coates *et al.*, 1955; Visek, 1978; Dibner & Richards, 2005). The overuse and misuse of antimicrobials quickly led to increased concerns over antimicrobial resistance, its potential transmission to humans, and the subsequent threat to public health (Smith *et al.*, 2002). In 2006, the use of sub-therapeutic levels of antimicrobials as AGPs was banned altogether by the European Union (Dibner & Richards, 2005; European Commission, 2005). Research has since focused on finding safe and viable alternative options for the replacement of AGPs in animal feeds.

Phytogenic or plant-derived feed additives have gained much attention for their potential to replace AGPs. They are known to have antimicrobial, antifungal, antiviral, antioxidant, and/or anticoccidial properties, which all promote gut health by beneficially modulating GIT microflora and by controlling potential pathogenic micro-organisms (Murugesan *et al.*, 2015). This in turn reduces immune stress in the animal, subsequently increasing the availability of energy and absorbed essential nutrients for growth and production (Windisch *et al.*, 2008). It has also been suggested that phytogenic feed additives (PFAs) have the ability to enhance nutrient utilization by promoting enzymatic activity and the production of digestive secretions in the GIT (Windisch *et al.*, 2008; Murugesan *et al.*, 2015). The beneficial effects of PFAs in the GIT is primarily accredited to the quality and quantity of their bioactive ingredients (Murugesan *et al.*, 2015).

Propolis is one example of a PFA that has been shown to promote gut health and also reduce immune stress in broilers. Propolis is a resinous product produced by bees to protect the hive, their larvae and themselves from harmful micro-organisms (Banskota *et al.*, 2001). Numerous studies have shown significantly higher antibody response and lymphoid organ relative weights in propolis-fed broilers, in both normal and heat stress environments (Hosseini *et al.*, 2016; Attia *et al.*, 2017; Chegini *et al.*, 2017; Zafarnejad *et al.*, 2017). These results indicated that immune function was improved in supplemented birds, as their ability to resist the effect of immunosuppression on lymphoid organ development was heightened. In terms of gut health, Abdel-Mohsein *et al.* (2014) reported that propolis-based diets showed a significant reduction of total anaerobes and coliform bacteria within the GIT of broilers that were subjected to heat stress.

The relative weights of the spleen and bursa of Fabricius are often used as a measure to predict the immune status of broiler chickens, as these lymphoid organs play a key role in defending the body against harmful micro-organisms (Cooper *et al.*, 1966; Abdel-Fattah *et al.*, 2008). Differences in relative weights may be related to changes in the efficiency or functionality of lymphoid organs (Cooper *et al.*, 1966). Relative lymphoid organ weights should, however, not be used solely to predict immune response, as the lowest weight may not necessarily be linked to decreased lymphoid cell production. It has thus been recommended that these weights be correlated with other immune response parameters before coming to any final conclusions (Kabir *et al.*, 2004). Other measures of gut health and immune response which may be used, include gizzard erosion scores, intestinal pH and liver colour.

The gizzard, also known as the muscular stomach, is responsible for grinding up feed in the GIT of broilers, and is thus a very important digestive organ. The presence of lesions in the gizzard make digestion painful, which consequently leads to a reduction in feed intake which subsequently suppresses growth (Contreras & Zaviezo, 2006). Gizzard lesions or gizzard erosion, can be caused by a number of factors, most of which can be related to the feed and/or poor gut health. Some of the leading causes of gizzard erosion include: the presence of mycotoxins in the feed; bacterial infections, i.e. caused by the clostridium species; fungal infections, i.e. caused by the aspergillus and fusarium species; viral infections, i.e. Infectious bronchitis virus or Newcastle disease; and nutritional causes, i.e. due to vitamin deficiency or excessive amounts of gizzerosine from high inclusion rates of fish meal (Contreras & Zaviezo, 2006). Gizzards are thus often scored according to a set scale so as to determine whether any test diets or ingredients have negatively affected this vital digestive organ.

The pH level at particular sites of the GIT is useful in predicting gut health as it is closely linked to the establishment of a specific microbial population. Intestinal pH also contributes to the degree of digestibility and absorptive value of most nutrients (Rahmani & Speer, 2005). Most pathogenic microbes grow in a pH of 7 or slightly higher, while beneficial micro-organisms thrive in a more acidic environment i.e. at a pH of 5.8 - 6.2 (Ford, 1974). A reduction in GIT pH can therefore inhibit the growth of acid-intolerant pathogenic bacteria such as *E. coli*, *Salmonella* and *Campylobacter* (Dibner & Buttin, 2002). At the same time, a more acidic environment inhibits microbial proliferation, thereby reducing growth-depressing microbial metabolites such as ammonia and amines; while also minimizing the competition between microflora and the host animal for nutrients (Dibner & Buttin, 2002).

The appearance and colour of the liver may also aid in defining the gut health and immune status of a bird. When a chicken hatches, the lobes of the liver have a bright yellow colour due to the pigments and lipids that are absorbed from the egg yolk. From day 8 to 14 in the chick's lifecycle, the liver colour gradually changes to the distinctive mahogany red-brown colour, characteristic of an adult chicken liver. The colour of the liver is thus dependent on the bird's nutritional state (McLelland, 1990). Pale and/or yellow discolorations of adult livers have been observed in studies where birds were exposed to nutritional stressors, such as: mycotoxins in the feed (Hoerr *et al.*, 1982; Kumar & Balachandran, 2009) and high fat diets (Butler, 1976; McLelland, 1990). Liver discolorations are also often accompanied by fatty degeneration, liver necrosis and/or haemorrhaging (Aengwanich & Simaraks, 2004; Kumar & Balachandran, 2009).

This study was conducted to investigate whether the use of the phytogenic feed additive, VivoCare® had any effects on the gut health and immune status of broiler chickens. Parameters that were measured included gizzard erosion scores, organ relative weights, intestinal pH and liver colour. The AGP-free negative control was unofficially accepted as the "worst-case scenario", while the positive control (with the commercially used and available AGP, Zinc Bacitracin) was the "best-case scenario".

4.2. Materials and methods

The particulars regarding the experimental animals, layout and treatments for the main trial (primary trial) are described in Chapter 3. In summary, five treatment diets, each replicated six times, were fed *ad libitum* to broiler chickens according to a completely randomised design. The treatments consisted of a negative control (NEG), a positive control (POS), and three diets which each contained a different concentration of the phytogenic feed additive, VivoCare®. These three inclusion levels were 500, 600 and 800 mg of VivoCare® per kg of feed and are further referred to as P500, P600 and P800, respectively. The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd after studying signal molecules and gene expression in different types of propolis, and it contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components.

For sampling, one representative bird from each cage was selected at day 14 and day 35. Birds were sacrificed by means of cervical dislocation on day 14; and were slaughtered according to standard commercial procedures by means of electrical stunning (50 - 70 volts; 3 - 5 seconds), followed by exsanguination within 10 seconds of stunning, at day 35.

4.2.1. Gizzard erosion trial

In addition to the primary trial as described in Chapter 3, another smaller gizzard erosion trial (secondary trial) was conducted simultaneously in the same housing system. The aim of this trial was to determine whether higher inclusion levels of VivoCare® in the diet caused gizzard erosion. Treatments consisted of a diet containing the highest inclusion level VivoCare® per kg of feed from the main trial i.e. P800; a diet with double the latter mentioned dosage (P1600); and a positive and negative control diet matching those from the primary trial. A summary of these treatment diets is presented in Table 4.1. For the purpose of this gizzard erosion trial, 40 chicks were used with ten chicks per cage and one cage per treatment. For the first seven days, all chicks received a control diet (equivalent to that used in the primary trial); thereafter, the test diets were fed for a further seven days. At day 14, all birds were sacrificed by means of cervical dislocation and the gizzards removed for scoring. Gizzards were cut open longitudinally, cleaned beneath running water and scored from 1 to 5 according to the gizzard erosion ordinal scale seen in Table 4.2. Gizzards from the main trial were also scored according to this scale on days 14 and 35.

Table 4.1 Description of treatments used from day 7 to day 14 for the Gizzard erosion trial

Treatment	Inclusion	Description
P800	800 mg/kg of feed	Treatment with highest inclusion level of phytogenic product from Primary trial (Beonics Feed Supplements (Pty) Ltd)
P1600	1600 mg/kg of feed	Double the inclusion of the P800 treatment
POS	150 mg/kg of feed	Positive Control (AGP[1] – Zinc Bacitracin)
NEG	No AGP[1]	Negative control

[1]Antimicrobial growth promoter – Zinc Bacitracin

Table 4.2 Gizzard erosion scoring and description

Score	Description
1	No erosion
2	Light erosion (roughness of epithelia)
3	Modest erosion (roughness and gaps)
4	Severe erosion (roughness, gaps, and ulcers, on stomach wall showing slight haemorrhaging)
5	Extreme erosion (roughness, gaps and haemorrhagic ulcers on stomach wall and separation of epithelia from stomach wall)

4.2.2. Organ data

The gizzard, liver, heart, spleen and bursa of Fabricius were removed from the fresh carcass, cleaned of any excess fat and weighed using a Mettler PC 4400 laboratory scale (Mettler-Toledo, Switzerland). When comparing treatment effects, the absolute weights, relative weights as a percentage of live body weight and the spleen to bursa ratio were evaluated.

After the organs were removed, pH measurements were taken using a calibrated (standard buffers pH 4.0 and 7.0 at 25°C) portable Crison pH 25 meter (Alella, Barcelona) at five regions of the digestive tract within 15 minutes post mortem. These points of measurement include the approximate centre of the proventriculus, duodenum, jejunum, ileum and caecum. After each reading was taken, the probe was rinsed thoroughly with distilled water.

The colour of the liver (L*, a* and b* measurements) was measured using the BYK-Gardner Colour Guide with colour described according to the CIE-Lab system using a CIE-Lab colour meter (BYK-Gardner GmbH, Gerestried, Germany) where L*, a* and b* represented lightness, redness and yellowness, respectively. Colour readings were taken at three randomly selected locations on each liver and averages were then calculated.

4.2.3. Statistical analysis

All statistical procedures were performed using SAS® statistical software (SAS Enterprise Guide 2014, Version 7.1, SAS Institute Inc., Cary, NC, USA). Based on the fact that the gizzard erosion data was ordinal, scores were displayed in the form of a contingency table and analysed using Fisher's exact test. Chi-squared *P* values were then used to evaluate differences where if $P < 0.05$, difference were significant. For the remaining parameters, the assumptions of homoscedasticity and normality of residuals was tested, and when satisfied, an Analysis of Variance (ANOVA) was carried out by means of the general linear model (GLM) procedure. Differences were said to be significant when $P < 0.05$. If significant differences existed, multiple comparison *post-hoc* tests were performed using Bonferroni (Dunn) t Tests.

4.3. Results and discussion

4.3.1. Gizzard erosion trial

The gizzard erosion scores for the primary trial at day 14 and 35, and the secondary trial at day 14, are given in Table 4.3 and Table 4.4, respectively. It was seen that in both trials there were no significant differences ($P > 0.05$) in gizzard erosion scores between any of the experimental diets. Incidences of severe or extreme gizzard erosion were absent in all cases, except that of a single bird from the negative control group at day 35 of the primary trial.

The causative agents of gizzard erosion include: mycotoxins; bacterial, fungal and viral infections; as well as nutritional factors such as vitamin deficiencies and high concentrations of gizzerosine from fish meal in the diet (Contreras & Zaviezo, 2006). For this study, it was concluded that none of the diets (even the double dose

treatment P1600) contained sufficient amounts of any causative agents to result in significant severe gizzard erosion which would have negatively affected production.

Table 4.3 Number of observations per category of gizzard erosion scores recorded for broilers of the primary trial at 14 and 35 days after receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Day	Gizzard erosion score				
		1	2	3	4	5
P500	**14**	2	3	1	0	0
	35	5	0	1	0	0
P600	**14**	2	3	1	0	0
	35	6	0	0	0	0
P800	**14**	4	1	1	0	0
	35	4	2	0	0	0
POS	**14**	2	3	1	0	0
	35	6	0	0	0	0
NEG	**14**	4	2	0	0	0
	35	3	2	0	1	0
Chi-Square *P* value	**14**			0.911		
	35			0.134		

[1]Antimicrobial growth promoter; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 4.4 Number of observations per category of gizzard erosion scores recorded for broilers of the secondary trial at 14 days of age after receiving a normal (P800) and double dosage of VivoCare® (P1600), versus a positive and negative control

Treatment	Gizzard erosion score				
	1	2	3	4	5
P800	2	6	2	0	0
P1600	2	7	1	0	0
POS	3	7	0	0	0
NEG	3	7	0	0	0
Chi-Square *P* value			0.851		

P800: 800 mg of VivoCare® per kg of feed; P1600: 1600 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

4.3.2. Organ weights

The gizzard, heart and liver absolute average weights and percentage of body weight at day 14 and 35 are presented in Table 4.5 and Table 4.6, respectively. The weights and percentages for these organs were statistically similar (P >0.05) for all of the experimental diets at both day 14 and day 35. These results are in agreement with other studies which have tested the effects of propolis ethanol extracts on broiler organ weights under normal conditions, as well as in heat stressed broilers (Daneshmand *et al.*, 2015; Hosseini *et al.*, 2016; Gheisari *et al.*, 2017; Zafarnejad *et al.*, 2017).

The average absolute weights of the spleen and bursa of Fabricius at day 14 and day 35 are given in Table 4.5. The weights of these lymphoid organs were further expressed as relative percentages of body weight, as well as in the form of a ratio, (i.e. the spleen:bursa ratio) as given by Table 4.7. At day 14, no differences (P >0.05) were observed between the experimental diets for the spleen:bursa ratio, the spleen and bursa absolute average weights, or for these organs as a percentage of body weight. At day 35, the bursa average absolute weights and percentage of body weight, as well as the spleen: bursa ratios, were statistically similar (P >0.05) for all of the experimental diets. Differences (P <0.05) were, however, observed for the spleen absolute average weights and percentage of body weight at day 35. Birds fed diet P800 had significantly smaller absolute spleen weights than birds in the P600 and negative control group. When these results were standardised by expressing their weights as a percentage of body weight, it was seen that group P800 had significantly smaller relative spleen weights in comparison to the negative control group.

A reduction in relative lymphoid organ weight has been associated with reduced immune functionality or efficiency of these lymphoid organs (Abdel-Fattah *et al.*, 2008). The results from this study might therefore have implied that the smaller spleen size of birds fed diet P800 indicated that the immune status and functionality of these birds were not as advanced as those of the negative control. In contrast, large lymphoid organs have also been associated with immunological stress whereby some form of mild infectious challenge stimulated the constant activation of the immune system (Kidd, 2004). This in turn could have suppressed growth and performance, as preference for nutrient and energy utilization was given to the immune response under these challenging conditions (Benson *et al.*, 1993; Kidd, 2004). This could have, in fact, been the case in this study, as in Chapter 3 it was seen that the average overall feed intake, live weight and live weight gain for the negative control birds were indeed the lowest in comparison to the other experimental diets.

Other studies that have used propolis ethanol extracts in broiler feeds indicated that this additive predominantly increased or had no effect on relative lymphoid organ weights in comparison to the negative control (Eyng *et al.*, 2015; Hosseini *et al.*, 2016; Zafarnejad *et al.*, 2017). Further investigation is thus required to make more definite assumptions about the relative spleen weight differences found in this study and its subsequent connection to immune response.

Table 4.5 Average organ weights (± standard error) for broilers slaughtered at 14 and 35 days of age receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Day	Treatment	Gizzard (g)	Heart (g)	Liver (g)	Spleen (g)	Bursa (g)
14	**P500**	13.5 ± 0.8	3.8 ± 0.2	16.3 ± 0.8	0.5 ± 0.1	1.1 ± 0.1
	P600	14.1 ± 0.8	4.2 ± 0.2	16.6 ± 1.0	0.5 ± 0.1	1.3 ± 0.1
	P800	14.1 ± 0.5	3.7 ± 0.2	16.2 ± 0.4	0.5 ± 0.0	1.5 ± 0.1
	POS	14.2 ± 0.8	4.0 ± 0.1	15.7 ± 0.3	0.5 ± 0.0	1.4 ± 0.2
	NEG	14.1 ± 0.5	4.1 ± 0.2	16.7 ± 0.7	0.4 ± 0.0	1.3 ± 0.1
	***P* value**	0.966	0.317	0.865	0.795	0.356
35	**P500**	30.0 ± 1.4	9.3 ± 0.6	43.7 ± 2.1	1.8^{ab} ± 0.1	4.4 ± 0.3
	P600	28.8 ± 1.2	10.4 ± 0.7	46.7 ± 1.9	2.3^{a} ± 0.2	4.0 ± 0.4
	P800	29.3 ± 1.5	8.4 ± 0.3	42.2 ± 2.2	1.6^{b} ± 0.2	3.3 ± 0.2
	POS	28.8 ± 2.3	9.1 ± 0.4	43.5 ± 1.4	2.1^{ab} ± 0.1	4.5 ± 0.3
	NEG	30.2 ± 1.2	9.7 ± 0.5	43.4 ± 1.0	2.3^{a} ± 0.1	4.0 ± 0.4
	***P* value**	0.941	0.135	0.486	**0.008**	0.155

[a,b] Means within columns with different superscripts differ significantly (*P* <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 4.6 Average organ weight as a percentage of body weight (± standard error) for broilers slaughtered at 14 and 35 days of age receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Day	Treatment	Gizzard (%)	Heart (%)	Liver (%)
14	**P500**	3.01 ± 0.19	0.84 ± 0.04	3.61 ± 0.11
	P600	3.14 ± 0.17	0.93 ± 0.03	3.68 ± 0.13
	P800	3.25 ± 0.16	0.84 ± 0.03	3.76 ± 0.18
	POS	3.17 ± 0.14	0.89 ± 0.02	3.52 ± 0.10
	NEG	3.09 ± 0.10	0.89 ± 0.04	3.65 ± 0.13
	***P* value**	0.842	0.287	0.795
35	**P500**	1.38 ± 0.06	0.43 ± 0.03	2.01 ± 0.07
	P600	1.26 ± 0.05	0.45 ± 0.02	2.04 ± 0.03
	P800	1.44 ± 0.07	0.41 ± 0.02	2.06 ± 0.08
	POS	1.37 ± 0.10	0.43 ± 0.02	2.07 ± 0.07
	NEG	1.46 ± 0.05	0.47 ± 0.03	2.11 ± 0.09
	***P* value**	0.309	0.409	0.878

P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 4.7 Average lymphoid organ weight as a percentage of body weight (± standard error) and average spleen to bursa ratio (± standard error) for broilers slaughtered at 14 and 35 days of age receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Day	Treatment	Spleen (%)	Bursa (%)	Spleen:Bursa
14	**P500**	0.113 ± 0.016	0.25 ± 0.02	0.46 ± 0.07
	P600	0.111 ± 0.014	0.28 ± 0.03	0.42 ± 0.08
	P800	0.113 ± 0.014	0.34 ± 0.02	0.34 ± 0.05
	POS	0.105 ± 0.010	0.32 ± 0.04	0.35 ± 0.04
	NEG	0.091 ± 0.009	0.29 ± 0.02	0.32 ± 0.03
	***P* value**	0.708	0.275	0.370
35	**P500**	0.081^{ab} ± 0.005	0.20 ± 0.01	0.41 ± 0.04
	P600	0.100^{ab} ± 0.006	0.18 ± 0.02	0.59 ± 0.04
	P800	0.079 ^{b} ± 0.010	0.16 ± 0.01	0.50 ± 0.07
	POS	0.099^{ab} ± 0.005	0.21 ± 0.02	0.48 ± 0.04
	NEG	0.109^{a} ± 0.006	0.19 ± 0.02	0.59 ± 0.07
	***P* value**	**0.014**	0.203	0.115

^{a,b} Means within columns with different superscripts differ significantly (P <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

4.3.3. Intestinal pH

The average pH values at five different sites of the digestive tract for broilers slaughtered at 35 days of age receiving different concentrations of the phytogenic feed additive, *VivoCare*®, can be seen in Table 4.8. No significant differences (*P* >0.05) were found between the different experimental diets for pH values of the proventriculus, duodenum, jejunum, ileum or the caecum.

The pH values in this study were in agreement with other studies using Cobb 500 broiler chickens (de Sousa *et al.*, 2015; Nkukwana *et al.*, 2015). As there were no significant differences between treatment diets, it was assumed that VivoCare® did not have the ability to significantly influence intestinal pH, and consequently gut health, in either a positive or negative manner. Other studies that have tested different phytogenic additives (i.e. black cumin seeds and Artemisia leaves; propolis and bee pollen; and a plant extract based product, Ateli plus®) for their potential to replace AGPs have similarly indicated a lack of differences in intestinal pH (Khalaji *et al.*, 2011; Kročko *et al.*, 2012; Teuchert, 2014).

Table 4.8 Average pH values (± standard error) of various regions of the digestive tract for broilers slaughtered at 35 days of age receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Proventriculus	Duodenum	Jejunum	Ileum	Caecum
P500	3.09 ± 0.25	5.92 ± 0.04	5.76 ± 0.04	5.87 ± 0.31	6.40 ± 0.18
P600	3.54 ± 0.26	5.98 ± 0.03	5.87 ± 0.02	6.30 ± 0.12	6.26 ± 0.17
P800	3.08 ± 0.12	5.83 ± 0.04	5.73 ± 0.02	6.02 ± 0.31	6.51 ± 0.21
POS	3.47 ± 0.42	5.67 ± 0.15	5.74 ± 0.04	6.43 ± 0.16	6.57 ± 0.09
NEG	3.39 ± 0.29	5.88 ± 0.06	5.71 ± 0.06	6.18 ± 0.30	6.76 ± 0.18
***P* value**	0.696	0.083	0.051	0.563	0.333

P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

4.3.4. Liver colour

The average colour measurements (lightness, redness and yellowness) for the liver of broilers slaughtered at 14 and 35 days of age receiving different concentrations of the phytogenic feed additive, VivoCare®, can be seen in Table 4.9. At day 14, no differences (*P* >0.05) were observed between experimental diets for any of the colour measurements. At day 35, there were no differences (*P* >0.05) between treatments for the measurements lightness and redness, however, significant differences did occur for yellowness.

The liver of the negative control birds at day 35 had the highest b* (yellowness) values, which were significantly more yellow than birds fed the P600 diet. Although this difference in yellowing was not noticeably obvious, and there were no visible signs of liver necrosis or haemorrhaging, it was speculated that the difference could have been a sign of early or mild liver stress. Liver damage and discolouration can be caused by a variety of

physiological disturbances, dietary defects and toxic substances which can often lead to the accumulation of fat in the liver, known as fatty degeneration (Butler, 1976). Fatty degeneration in the liver has a characteristic swollen, yellow and greasy appearance; and under microscopic examination, cells appear enlarged, pale and lacy (Aengwanich & Simaraks, 2004). Further investigation is needed to confirm the results and speculations regarding liver colour in this particular study. If VivoCare® truly does have the potential to prevent fatty degeneration, it may aid in the prevention of fatty liver haemorrhagic syndrome, a metabolic condition which is a major cause of death in caged layer commercial flocks (Shini & Bryden, 2009).

Table 4.9 Average (± standard error) colour measurements (CIE-Lab) of livers from broilers slaughtered at 14 and 35 days of age receiving different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Day	Treatment	L* (lightness)	a* (redness)	b* (yellowness)
14	**P500**	35.62 ± 1.20	9.63 ± 1.76	11.86 ± 1.57
	P600	33.45 ± 1.03	10.64 ± 1.40	10.91 ± 0.60
	P800	36.07 ± 1.11	12.75 ± 1.69	9.24 ± 2.38
	POS	33.81 ± 1.40	11.29 ± 1.65	9.24 ± 1.56
	NEG	36.38 ± 1.31	9.42 ± 1.49	12.79 ± 1.11
	***P* value**	0.329	0.590	0.413
35	**P500**	31.91 ± 1.21	11.93 ± 0.34	11.74^{ab} ± 0.90
	P600	28.91 ± 0.81	12.80 ± 0.38	9.52^{b} ± 0.59
	P800	28.77 ± 0.67	12.65 ± 0.41	10.06^{ab} ± 0.25
	POS	33.77 ± 2.67	11.81 ± 1.16	12.43^{ab} ± 0.65
	NEG	32.18 ± 1.14	13.00 ± 0.25	12.89^{a} ± 0.99
	***P* value**	0.087	0.548	**0.006**

[a,b] Means within columns with different superscripts differ significantly (*P* <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

4.4. Conclusion

The results from this study showed that VivoCare® did not cause any significant gizzard erosion, even when inclusion levels were doubled. It also had no significant effect on GIT pH. It was speculated that the significantly larger spleen relative weights of the negative control birds in comparison to P800 fed birds at day 35, could have been related to a more developed immune response. It was also possible that the energy and nutrients that were used to create this advance in immunity, would otherwise have been used for growth and production. These effects may have been contributing factors to the reduced feed intake and body weight gain seen for the negative control birds in Chapter 3. The significantly higher scores for liver yellowness in the negative

control birds (compared to P600 birds) could have potentially been related to a mild form of liver stress, however, the lack of visibly noticeable discolouration, haemorrhaging and necrosis, indicated that the challenge was not severe enough to allow for obvious conclusions. Overall, there was little evidence to suggest that there were any major differences in gut health and immune status between the experimental groups. The negative control did seem to show slightly more evidence of exposure to immunological stress, however, differences were not prominent and other measures of gut health and immune response should be further investigated to better support these results. Future studies should additionally investigate the histomorphology of the GIT (i.e. the villi height and crypt depth); the blood constituents (i.e. lipid concentrations in the serum and antibody titer); and liver and kidney samples for microscopic examination; so as to get a closer look at the effects of VivoCare® in the broiler chicken at the cellular level. Investigating these parameters in an environment where a deliberate stressor is introduced would also add great value, as this would demonstrate the ability of VivoCare® to resist the negative effects of such a challenge; which is an effect that AGPs are well known for.

4.5. References

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Chapter 5

The effect of a phytogenic feed additive on the carcass characteristics and skeletal parameters of broiler chickens

Abstract

This study was performed to investigate the effect of the phytogenic feed additive, VivoCare®, on the carcass characteristics and skeletal parameters of mixed sex, Cobb 500 broiler chickens. VivoCare® is produced by Beonics Feed Supplements (Pty) Ltd, and contains bio-active components originally identified from propolis. A primary trial was conducted to investigate the carcass characteristics and a secondary trial to examine the skeletal parameters. The primary trial consisted of five diets, each replicated six times. These diets included a positive and negative control, and three test diets with VivoCare® included at 500, 600 and 800 mg/kg of feed (P500, P600 and P800). The same five diets were used for the secondary trial. In comparison to the negative control, VivoCare® had no significant effect on carcass weights; dressing percentage; carcass portion and breast component yields; breast and thigh pH; breast meat colour; tibia bone weight, length, diameter, breaking strength, fat free dry weight, ash percentage and mineral content. Noted significant differences include: P600 birds that had smaller dressing percentages than P500 birds; positive control birds that had significantly longer tibia bones compared to P800 birds; and higher tibia phosphorus content of P600 birds compared to the positive control. Significant correlations were noted between breast ultimate pH and breast b* (yellowness) measurements; tibia bone wet weight and bone length, diameter and breaking strength; and also between bone breaking strength and bone length. It was concluded that VivoCare® had no noteworthy effects on carcass quality or skeletal parameters in this study and additional research with this product was thus recommended.

Keywords: breaking strength, breast colour, caffeoylquinic acid, dressing percentage, meat pH, prodelphinidin, propolis, three point bending test

5.1. Introduction

There are several contributing factors that influence a consumer's initial selection and acceptance of a raw meat product in the marketplace (Fletcher, 1999a). The most prominent of these factors include; the meat colour, the amount of visible fat, and the presence of any visual defects usually associated with haemorrhaging, bone breakage or bruising (Le Bihan-Duval *et al.*, 1999; Fletcher, 2002).

In terms of meat colour and quality, a pale tan to pink coloured chicken breast fillet is generally preferred and accepted by consumers. The two extreme conditions in which meat is rejected, is when it is pale, soft, and exudative (PSE-like); or when it is dark, firm and dry (DFD-like) (Fletcher, 2002). Muscle colour and its corresponding pH are said to be highly correlated. Meat with a higher pH has been associated with a darker colour; while a lower pH has been associated with a lighter colour (Allen *et al.*, 1998; Fletcher, 2002).

Through the decades, selection criteria for broiler chickens have been altered to suite the increase in population growth and the accompanying change in market demand for chicken meat (Petracci *et al.*, 2015). In the last two decades there has been an increasing preference for breast meat, especially in Western countries, accompanied by the growing demand for further-processed meat products (Fletcher, 2002; Petracci *et al.*, 2015). This, along with the consumer desire for a healthier meat product, has led to selection for higher breast meat yields with less abdominal fat (Le Bihan-Duval *et al.*, 1999; Petracci *et al.*, 2015).

Visual defects can have a dramatic effect on the appeal of meat to a consumer (Fletcher, 2002). Discoloration from bruising, or the presence of blood from haemorrhages or bone breakage in the tissue or below the skin, have been a major source of product downgrading and condemnation (Bilgili & Hess, 1995; Fletcher, 2002; Driver *et al.*, 2006). With broiler breeding programs selecting for increased growth and body weights over the years, broilers have become especially susceptible to bone abnormalities, deformities and breakage (Rath *et al.*, 2000). Although these issues can be potentially eliminated or reduced by slower growth rates, bone disorders are not always related to heavy birds and rapid growth (Driver *et al.*, 2006; Waldenstedt, 2006). It has been said that these problems are generally more related to interactions between genetic, nutritional and management factors (Waldenstedt, 2006). Dietary calcium and phosphorous deficiencies have been shown to cause bone breakage and bloody breast meat during carcass processing (Driver *et al.*, 2006). Some other known contributing factors to these adverse effects on carcass quality include stress, light intensity, mycotoxins, housing density, gender, stunning and killing methods (Bilgili & Hess, 1995; Fletcher, 1999b, 2002; Olanrewaju *et al.*, 2006).

In the past, antimicrobial growth promoters (AGPs) have been used in broiler nutrition to promote production performance while simultaneously maintaining optimal health status. Their use could reduce product variation in terms of size and weight, and could combat the adverse effects of poor management on carcass and meat quality by acting as a substitute for hygiene-management practices (Liu *et al.*, 2005; Key & Mcbride, 2014). Since the use of antimicrobials as AGPs was banned by the European Union in 2006, efforts have turned to finding suitable alternatives to replace AGPs in animal feeds (Dibner & Richards, 2005; European Commission, 2005).

Propolis, a natural resin produced by honey bees to protect their larvae, themselves and their hive from harmful micro-organisms, is a product that has received much interest as a potential phytogenic alternative to AGPs (Banskota *et al.*, 2001). Studies have shown that the inclusion of propolis extracts at higher concentrations in the diet can significantly increase carcass yield, dressing percentage and relative breast meat weights in comparison to the negative control (Seven *et al.*, 2008; Attia *et al.*, 2014; Zafarnejad *et al.*, 2017). At lower concentrations, propolis inclusion has been seen to have no significant effects on carcass and meat yields (Daneshmand *et al.*, 2015; Gheisari *et al.*, 2017). With regards to post mortem pH and meat colour, differences were not observed between propolis tested diets and the control (Šulcerová *et al.*, 2011; Haščík *et al.*, 2015). Studies have also demonstrated that propolis has the ability to accelerate bone tissue regeneration (Stojko *et al.*, 1978; Toker *et al.*, 2008), and to significantly improve the utilization and availability of dietary calcium and phosphorous in anaemic rats (Haro *et al.*, 2000). Caffeic acid phenethyl ester (CAPE), a bioactive component found in propolis, was also said to have potential in treating bone lytic diseases in humans (Ang *et al.*, 2009).

This study was conducted to investigate the effects of the phytogenic feed additive, VivoCare®, on carcass, meat and skeletal parameters. Parameters that were measured included: warm and cold carcass weights, dressing percentage, carcass and breast component yields, post mortem thigh and breast pH, breast meat colour (CIE-Lab), tibia breaking strength, and bone ash and mineral content. Correlation tests were also conducted for breast pH and colour; as well as for some of the bone parameter measurements. The trial was conducted with both a positive (the commercially used and available AGP, Zinc Bacitracin) and a negative control (AGP-free diet) so as to make adequate final comparisons.

5.2. Materials and methods

5.2.1. Carcass characteristics

The details regarding the experimental animals, layout and treatments are described in Chapter 3. In summary, five treatment diets, each replicated six times, were fed *ad libitum* to broiler chickens according to a completely randomised design. The treatments consisted of three diets each with a different inclusion level of VivoCare® (500, 600 and 800 mg/kg of feed), as well as a positive (with Zinc bacitracin as an AGP) and negative (no AGP) control diet. The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd after studying signal molecules and gene expression in different types of propolis, and it contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components.

One representative bird from each cage was selected at day 35 to assess the carcass characteristics. The live weights were measured using an LBK ADAM® scale (Adam Equipment Co Ltd, Kempton Park, Johannesburg) and recorded. Birds were subsequently slaughtered according to standard commercial procedures by means of electrical stunning (50-70 volts; 3-5 seconds), followed by exsanguination within 10 seconds of stunning. Carcasses were then scalded, de-feathered and eviscerated; whereby the feet, neck and all internal organs were removed.

The initial (pH_I) and ultimate pH (pH_U) were measured for both the breast and thigh muscle using a calibrated portable Crison pH25 meter (Alella, Barcelona). The pH_I was determined 15 minutes post mortem, while the pH_U was taken 24 hours post mortem in the same position and manner as for the pH_I. The procedure involved making a small incision in the centre of the breast or thigh muscle where after the probe was inserted and positioned so as to obtain a stable and accurate reading.

After the pH_I had been measured, the carcass was weighed (LBK ADAM® scale) and the warm carcass weight recorded. All carcasses were subsequently placed in cold storage at 4°C for 24 hours and then weighed again (LBK ADAM® scale) to determine cold carcass weight. Once weighed, cold carcasses were divided into commercial portions using a portion cutter. This involved cutting the carcass in two, after which the thigh and drumstick were removed by cutting directly above the thigh towards the acetabulum just behind the pubic bone. To separate the drumstick and thigh, this meat portion was severed perpendicular to the joints where the tibia and fibula meet the femur. The wing and breast were divided by cutting through the joint between the scapula and coracoid. Additionally, the breast portion was further separated into three parts, namely; muscle, skin with subcutaneous fat, and bone. All of the components were weighed using a Mettler PC 4400 scale (Mettler-

Toledo, Switzerland) and recorded. The dressing percentage, portion yield percentage and breast component percentage yield were calculated according to Equation 5.1, Equation 5.2 and Equation 5.3, respectively.

Equation 5.1: $$\text{Dressing percentage} = \frac{\text{Warm carcass weight (g)}}{\text{Live weight (g)}} \times 100$$

Equation 5.2: $$\text{Portion yield percentage} = \frac{\text{Portion weight (g)}}{\text{Cold carcass weight (g)}} \times 100$$

Equation 5.3: $$\text{Breast component yield (\%)} = \frac{\text{Breast component (g)}}{\text{Total breast weight (g)}} \times 100$$

The colour of the breast meat (L*, a*, b* measurements) was measured using a CIE-Lab colour meter (BYK-Gardner GmbH, Gerestried, Germany), where L*, a* and b* represent lightness, redness and yellowness, respectively. Before readings were taken, the skin of the breast was removed and the breast allowed to bloom for 15 minutes (Warriss, 2000) at 8°C on a flat surface. Colour readings were then taken in triplicate at randomly selected locations on each breast and averages were calculated. Correlations between the meat pH and colour were also investigated using statistical analysis.

5.2.2. Skeletal parameters

For the purpose of collecting bone data, a separate secondary trial was conducted in the same environmentally controlled housing system with the same treatment diets as used in the primary trial (Chapter 3). For the purpose of this secondary trial, 80 chicks were used with ten chicks per cage and one cage per treatment. Chicks were fed *ad libitum* and were all sacrificed at day 35 according to standard commercial practices which involved electrical stunning (50-70 volts; 3-5 seconds), followed by exsanguination within 10 seconds of stunning.

Both tibias were removed, bagged and frozen at -20°C until further analysis. The left tibias were thawed overnight at 4°C, cleaned of all adherent tissue and weighed. Tibia length and mid diaphyseal diameter were measured using a Vernier calliper with accuracy of 0.01 mm. The centre point of the tibia was marked with ink and the bones then refrigerated until the breaking procedure. Tibia breaking strength was tested using a three point destructive bending test (Fleming *et al.*, 1998), performed by an Instron tensile/compression machine with a 5 kN load cell capacity. Tibias were placed in a stable position on two retaining bars with the ink-marked centre point located directly below the breaking probe. Each retaining bar was 10 mm in diameter and was set 40 mm apart. The probe crosshead (also 10 mm in diameter) approached the tibia centre point at a constant speed of 30 mm/min until the bone was broken. The Instron machine was controlled by means of a computer using a HBM MVD25010 signal conditioning and data acquisition system. This computer program was used to record the force (N) and corresponding displacement every 0.02 seconds during the breaking procedure. The breaking strength was taken as the point of maximum load before failure and was given in absolute force (N). Correlations between the bone length (mm), diameter (mm) and breaking strength (N) were also investigated using statistical analysis.

The right tibias were thawed overnight at 4°C, cleaned of all adherent tissue and weighed. Dry matter for each tibia was subsequently determined according to the Official Method 934.01 of the AOAC (AOAC International,

2002). Tibias were dried at 100°C for 24 hours in porcelain crucibles. They were then removed from the drying oven and allowed to cool in a desiccator for 30 min, where after the dry tibia weight was determined. The dried tibias were broken in half and defatted for a period of 48 hours in petroleum ether. The tibias then underwent a second 24 hour drying session at 100°C to determine fat-free dry bone weight. Dried and defatted bone samples were finally ashed at 600°C for 24 hours and the fat free bone ash percentage determined. All bone related weights were measured using a Mettler AE 200 scale (Mettler-Toledo, Switzerland) with 0.0001 g accuracy. Once removed from the ash oven, the tibia ash remains were ground to a fine powder using a mortar and pestle. The ash samples were then sent for mineral analysis to the Institute of Animal Production, Western Cape Department of Agriculture at Elsenburg. Mineral composition was determined according to Combustion Method No. 6.1.1 as described by the Agriculture Laboratory Association of Southern Africa (AgriLASA, 2007). In brief, 5 ml of 6 M hydrochloric acid was added to 0.5 g sample. Samples were then placed in an oven at 50°C for 30 min. Once removed from the oven, 35 ml distilled water was added and the solution filtered into a 50 ml bottle. Distilled water was then used to top up the solution to the 50 ml mark. The minerals were measured using an iCAP 6000 Series Inductive Coupled Plasma (ICP) Spectrophotometer (Thermo Electron Corporation, Strada Rivoltana, 20090 Rodana, Milan, Italy) fitted with a vertical quartz torch and Cetac ASX-520 autosampler. Finally, mineral concentrations were calculated using iTEVA Analyst software.

5.2.3. Statistical analysis

All statistical procedures were performed using SAS® statistical software (SAS Enterprise Guide 2014, Version 7.1, SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was conducted to test for the non-normality of residuals (Shapiro & Wilk, 1965), and Bartlett and Levene's tests were performed to test for heteroscedasticity (Bartlett, 1937; Levene, 1960). With the latter assumptions satisfied, Analysis of Variance (ANOVA) was carried out by means of the general linear model (GLM) procedure. Differences were said to be significant when P <0.05. If significant differences existed, multiple comparison *post-hoc* tests were performed using Bonferroni (Dunn) t Tests. Pearson's correlation coefficients (r) and the corresponding P values were used to investigate potential relationships between the parameters. Correlations were significant when P <0.05 and the correlation measures (r) were strongest when they approached the values 1 or -1.

5.3. Results and discussion

5.3.1. Dressing percentage and carcass portion yield

The average live weights (obtained from Chapter 3), warm carcass weights and dressing percentages from broilers slaughtered at 35 days of age, receiving different concentrations of the phytogenic feed additive, VivoCare® can be seen in Table 5.1. The carcass was divided into four portion components, namely; the breasts and back, the thighs, the legs and the wings. The yields were then expressed as a percentage of the cold carcass weight and these results can be seen in Table 5.2. The components of the breast (i.e. the muscle, skin with fat, and bone) were also measured and given as a percentage of the breast and back weight, and are given in Table 5.3.

No differences (*P* >0.05) existed between the treatment diets for warm or cold carcass weight, carcass portion yields, or for the breast component yields. Similar results were seen by Kleczek *et al.* (2014) who tested the effects of a chemically standardised form of propolis (10 and 50 mg/kg) on broiler carcass and meat yields. In contrast, a study by Seven *et al.* (2008), who investigated the effect of propolis on heat stressed birds, showed that a higher propolis inclusion level (1000 mg/kg) had no effect on relative leg, wing, neck and back weights; however, the breast meat weights were significantly higher than the negative control. Propolis may thus have a more profound effect on broiler meat yield in environments where some form of stressor is applied.

The one difference (P <0.05) that was observed in this study, was in dressing percentage between two of the VivoCare® diets, P500 and P600, whereby diet P600 birds had a significantly lower dressing percentage than diet P500 birds. High dressing percentage values are usually associated with larger body weights at slaughter (Attia *et al.*, 2014; Zafarnejad *et al.*, 2017). The results in Chapter 3, however, indicated that the body weights at day 35 of all the treatment diets were statistically similar (*P* =0.302). The lack of differences (*P* >0.05) between the experimental diets for warm carcass weight, means that the reduced dressing percentage of P600 birds, can also not be attributed to significantly smaller warm carcass weights. It was possible that the difference arose due to a combination of slightly heavier live weights with slightly reduced warm carcass weights for P600 birds.

Some known factors that influence the dressing percentage of broilers include: diet, gender, live weight, fat content and degree of feathering (Alberta, 2000; Zerehdaran *et al.*, 2004; Sakomura *et al.*, 2005). The factor that was of particular interest in this case was fat content. Dressing percentages can be higher in fatter birds, due to an increase in subcutaneous and intramuscular fat, which increases the carcass weight in relation to the live weight (Zerehdaran *et al.*, 2004). Havenstein *et al.* (2003) indicated that the fat content in broilers (at 43 days of age) represented as much as 10 to 15% of the total carcass weight. It was possible that the P600 birds had a lower carcass fat content, which subsequently resulted in a slightly reduced warm carcass weight, which in turn contributed to the drop in dressing percentage. There was evidence from Chapter 4 to support this claim, as P600 birds had significantly lower scores for liver yellowness at Day 35. As almost all fatty acid synthesis occurs in the liver of avian species, excessive lipogenesis will increase the rate of fat accumulation in adipose tissue and may result in liver fatty degeneration, which can be identified by a characteristic swollen, yellow and greasy appearance of the liver (Butler, 1976; Aengwanich & Simaraks, 2004). As the livers of P600 birds were significantly less yellow, it was speculated that this dietary treatment could have contributed to the inhibition of hepatic lipogenesis and fatty degeneration, which in turn reduced fat deposition in the carcass. Reduced fat content in meat has been associated with a healthier and higher quality meat product by modern day consumers and selection by producers for reduced fat content has thus become favoured over selection for increased dressing percentage (Le Bihan-Duval *et al.*, 1999). Further research is recommended to properly investigate these speculations, especially as none of the dietary treatments differed significantly from the negative control. If the speculations from this trial are indeed true, this additive may have great potential economic value.

With regards to other natural feed additives tested in broiler nutrition, a review by Fouad and El-Senousey (2014) reported that there are numerous phytogenic feed additives which demonstrated the ability to inhibit

hepatic lipogenesis and reduce abdominal fat percentage in broilers. Some examples of these additives include the alfalfa extract, polysavone (Dong *et al.*, 2007); green tea powder (Biswas & Wakita, 2001); ginseng (Qureshi *et al.*, 1983; Yan *et al.*, 2011); extracts from thyme and cinnamon (Al-Kassie, 2009; Abdulkarimi *et al.*, 2011); and propolis (Daneshmand *et al.*, 2015). The principle bioactive ingredients identified for these additives include polyphenolic compounds and saponins, which are both key active ingredients in propolis.

Table 5.1 Average (± standard error) live weight, warm carcass weight and warm dressing percentage from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Live weight[2] (g)	Warm carcass weight (g)	Dressing percentage
P500	2213.0 ± 37.7	1611.2 ± 57.8	69.96[a] ± 0.36
P600	2255.0 ± 21.7	1594.7 ± 31.7	66.93[b] ± 0.35
P800	2215.4 ± 31.3	1530.2 ± 37.8	69.47[ab] ± 0.95
POS	2218.4 ± 31.2	1619.2 ± 67.1	68.66[ab] ± 0.65
NEG	2159.3 ± 26.5	1472.7 ± 55.7	68.04[ab] ± 0.60
***P* value**	0.302	0.243	**0.017**

[a,b] Means within columns with different superscripts differ significantly (P <0.05); [2]Results obtained from Chapter 3; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 5.2 Average (± standard error) cold carcass weight and carcass portion percentage yields from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Cold carcass weight (g)	Breast and back (%)	Thigh (%)	Leg (%)	Wing (%)
P500	1594.2 ± 54.1	45.34 ± 0.70	28.08 ± 0.50	13.68 ± 0.33	10.01 ± 0.20
P600	1578.6 ± 30.0	44.71 ± 1.75	27.73 ± 1.05	14.05 ± 0.21	10.41 ± 0.38
P800	1510.3 ± 35.5	45.91 ± 1.27	28.02 ± 0.49	14.54 ± 0.46	9.75 ± 0.32
POS	1597.4 ± 64.7	45.70 ± 0.69	28.44 ± 0.48	14.03 ± 0.60	9.84 ± 0.44
NEG	1450.0 ± 56.2	46.12 ± 0.90	28.81 ± 0.48	13.88 ± 0.38	10.19 ± 0.23
***P* value**	0.191	0.913	0.794	0.678	0.622

Differences are significant when P <0.05; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 5.3 Average (± standard error) breast component yields as a percentage of the breast weight from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	% Muscle	% Skin and fat	% Bone
P500	62.08 ± 0.95	7.71 ± 0.75	28.01 ± 1.31
P600	61.13 ± 1.15	8.15 ± 0.79	31.01 ± 0.62
P800	60.34 ± 1.39	9.11 ± 0.70	30.87 ± 1.55
POS	62.11 ± 1.47	7.59 ± 0.69	27.75 ± 0.96
NEG	60.13 ± 1.48	7.06 ± 0.68	31.64 ± 1.59
***P* value**	0.728	0.361	0.111

Differences are significant when $P < 0.05$; [1]Antimicrobial growth promoter; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

5.3.2. Meat pH and colour measurements

The average initial and ultimate pH (pH_I and pH_U) of the breast and thigh muscle of broilers that received different concentrations of the phytogenic feed additive, VivoCare®, can be seen in Table 5.4, while the average colour measurements (CIE-Lab) for the breast meat portions is presented in Table 5.5. There were no differences ($P > 0.05$) in the pH_I or pH_U for both the breast and thigh muscle for any of the experimental diets. Differences ($P > 0.05$) in the breast colour measurements also did not exist; with statistically similar lightness (L*), redness (a*) and yellowness (b*) values for all of the experimental diets.

Studies that tested the effect of propolis extracts on muscle pH and colour showed a similar lack of differences between the test diets and the control (Šulcerová *et al.*, 2011; Haščík *et al.*, 2015). The breast and thigh muscle pH values from this study fell within the expected range for normal broiler meat (Fletcher, 1999a; Šulcerová *et al.*, 2011). Based on results from three articles that compared light, normal and dark broiler breast meat from commercial processing plants, measurements for normal meat had L* readings that ranged from 45.6 to 55.1; a* readings from 1.7 to 2.2; and b* readings from 1.52 to 9.6 (Fletcher, 1999a; Van Laack *et al.*, 2000; Petracci *et al.*, 2004). The L* values from this study fell within the expected normal range, while the a* and b* values were substantially higher than the specified norm. Research conducted with turkeys showed that when carcass chilling was delayed, meat had a lighter, redder, and more yellow colour (Rathgeber *et al.*, 1999). It was possible that due to the manual slaughter procedure in this trial, the time frame from slaughter to chilling was delayed in comparison to the rapid processing and storage procedures that exist in commercial processing plants. This may have then been the reason for the colour deviations from the normal ranges mentioned earlier.

Table 5.4 Average (± standard error) pH_I and pH_U of breast and thigh muscle obtained from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	pH_I Breast muscle	pH_U Breast muscle	pH_I Thigh muscle	pH_U Thigh muscle
P500	5.92 ± 0.07	5.74 ± 0.04	6.18 ± 0.06	5.91 ± 0.07
P600	6.01 ± 0.08	5.68 ± 0.03	6.01 ± 0.06	5.85 ± 0.04
P800	6.07 ± 0.17	5.66 ± 0.03	6.18 ± 0.11	5.77 ± 0.06
POS	6.09 ± 0.05	5.68 ± 0.03	6.05 ± 0.04	5.79 ± 0.08
NEG	6.17 ± 0.10	5.67 ± 0.03	5.99 ± 0.08	5.80 ± 0.04
***P* value**	0.527	0.560	0.212	0.464

Differences are significant when $P < 0.05$; pH_I: Initial pH; pH_U: Ultimate pH; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 5.5 Average (± standard error) colour measurements (CIE-Lab) of breast meat from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	L* (lightness)	a* (redness)	b* (yellowness)
P500	51.23 ± 0.60	3.28 ± 0.34	11.34 ± 0.76
P600	50.93 ± 0.77	3.34 ± 0.26	11.22 ± 0.59
P800	51.96 ± 1.03	3.73 ± 0.64	11.80 ± 0.35
POS	52.43 ± 0.83	3.05 ± 0.46	11.80 ± 0.29
NEG	51.88 ± 1.07	3.14 ± 0.38	11.36 ± 0.49
***P* value**	0.758	0.830	0.894

Differences are significant when $P < 0.05$; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

The relationship between ultimate breast pH values and their colour measurements was evaluated using Pearson's correlation coefficients as seen in Table 5.6. It was noted that the only significant correlation that existed was a moderate negative correlation between breast pH_U and breast yellowness ($R = -0.434$; $P = 0.016$). This implied that a lower pH_U could be associated with a more yellow breast. Other studies have shown a similar correlation between pH_U and yellowness (Allen *et al.*, 1997, 1998; Rathgeber *et al.*, 1999). It has also been previously noted that breast meat pH and colour correlations are more established in studies where wide ranges of breast meat colour are evident (Fletcher, 2002). As the breast meat samples from this study were neither PSE-like nor DFD-like, but rather regarded as "normal" with no differences in colour and pH_U; the insignificant correlation between breast meat lightness and pH was expected.

Table 5.6 Pearson's correlation coefficients (*r*) and accompanying *P* values of significance (*P*) between breast ultimate pH values and colour parameters (lightness, redness and yellowness) for broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

		b* (yellowness)	a* (redness)	L* (lightness)
Breast ultimate pH	*r*	**-0.434**	-0.173	-0.292
	P	**0.016**	0.359	0.118
L* (lightness)	*r*	0.346	-0.360	
	P	0.061	0.051	
a* (redness)	*r*	0.113		
	P	0.552		

n=48; correlations are significant when *P* <0.05

5.3.3. Skeletal parameters

The wet weight, length, diameter and breaking strength of tibias from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic feed additive, VivoCare®, can be seen in Table 5.7. Differences (*P* >0.05) in tibia wet weight, diameter and breaking strength did not occur between any of the experimental diet groups. There were, however, differences (*P* <0.05) in tibia length; whereby the positive control group had significantly longer tibias than the P800 group. Tibial dyschondroplasia (TD) can cause bone deformities and bending, which may contribute to reduced bone length and bone weakness (Lynch *et al.*, 1992). The reduction in bone length of P800 birds could thus possibly be associated with increased cases or severity of TD in these birds during the trial. Further investigation is, however, recommended to investigate this difference.

Additionally, all of the dietary treatments were statistically similar to the negative control for the wet weight, length, diameter and breaking strength parameters. This lack of differences was in agreement with a study by Kleczek *et al.* (2012) who found that diets supplemented with a form of propolis extract standardised with 5% quercetin (at 250 mg/kg of feed), had no significant effect on shear strength values or physical properties of broiler tibias.

Table 5.7 The average (± standard error) wet weight, length, diameter and breaking strength of tibia bones for broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Wet weight (g)	Length (mm)	Diameter (mm)	Breaking strength (N)
P500	11.5 ± 0.38	88.1^{ab} ± 0.66	8.14 ± 0.28	360.18 ± 27.27
P600	11.6 ± 0.45	88.8^{ab} ± 0.84	7.63 ± 0.11	385.00 ± 19.39
P800	10.8 ± 0.45	86.9^{b} ± 0.93	7.71 ± 0.26	355.05 ± 14.69
POS	12.4 ± 0.34	90.8^{a} ± 0.69	8.05 ± 0.31	425.07 ± 28.86
NEG	11.5 ± 0.43	89.5^{ab} ± 1.10	7.65 ± 0.18	389.62 ± 18.92
***P* value**	0.100	**0.027**	0.409	0.206

^{a,b} Means within columns with different superscripts differ significantly (P <0.05); P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Pearson's correlation coefficients (r) and the accompanying P values (P) for wet weight, length, diameter and breaking strength of the tested tibia bones are presented in Table 5.8. It was seen that tibia wet weights had significant correlations with tibia bone length (r =0.761; P <0.001), diameter (r =0.513; P <0.001) and breaking strength (r =0.490; P <0.001), although these correlations were not very strong. A significant, moderate correlation also existed between tibia length and breaking strength (N) (r =0.500; P <0.001). These correlations implied that heavier tibia bones could be potentially associated with longer, thicker and stronger bone measurements. Furthermore, it was noted that a significant correlation existed between tibia length and breaking strength, but not between tibia diameter and breaking strength (r =0.271; P =0.057). This suggested that thicker bones were thus not necessarily stronger. Avian long bones are made up of a low density, trabecular bone mass, surrounded by compact, high density cortical bone which forms the outer bone layer or shell (Rath *et al.*, 2000; Jendral *et al.*, 2008). The thickness of these two bone types are known to vary independently of bone diameter. Total bone density and breaking strength is thus better explained by the ratio of trabecular to cortical bone thickness, rather than by total bone diameter (Jendral *et al.*, 2008).

Table 5.8 Pearson's correlation coefficients (*r*) and accompanying *P* values of significance (*P*) between wet weight, breaking strength, diameter and length of tibia bones for broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

		Breaking strength (N)	Diameter (mm)	Length (mm)
Wet weight (g)	*r*	**0.490**	**0.513**	**0.761**
	P	**<0.001**	**<0.001**	**<0.001**
Length (mm)	*r*	**0.500**	0.078	
	P	**<0.001**	0.591	
Diameter (mm)	*r*	0.271		
	P	0.057		

n = 50; correlations are significant when *P* <0.05

Tibias were also analysed to obtain the fat free dry weight; ash content; calcium and phosphorous as a percentage of the ash content; and the calcium to phosphorous ratio (Ca:P); which can be seen in Table 5.9. Significant differences in fat free dry weight, calcium content and the Ca:P ratio did not exist (P >0.05). For the tibia bone ash content, the ANOVA indicated that significant differences existed between the treatment groups (P <0.05), however, after *post-hoc* comparison tests (Bonferroni t Tests) were performed, differences became insignificant between the treatment groups and it was decided that the results for ash content would be accepted as statistically similar. A difference (P <0.05) in percentage phosphorous content of the bone ash was seen, whereby diet P600 birds had a significantly higher bone phosphorous content in comparison to the positive control.

Together, calcium and phosphorus makes up between 60 and 70% of the bone mineral matrix; constituting roughly 370 and 170 g/kg bone ash, respectively (Doyle, 1979; Rath *et al.*, 2000). This translates to a Ca:P ratio of 2.17, of which results from this study were in agreement. An imbalance, due to an abnormal ratio between these nutrients, may be as detrimental as a dietary deficiency of either mineral (Waldenstedt, 2006). This, in turn, can contribute to an increase in the incidences and severity of tibial dyschondroplasia and bone fractures (Rath *et al.*, 2000; Waldenstedt, 2006).

Calcium and phosphorus are exchanged continuously between the soft tissue and bones (Rath *et al.*, 2000). Researchers have shown that propolis as a feed additive, can increase phosphorus levels in bones such as the femur and sternum (Haro *et al.*, 2000); and can decrease serum phosphorus in the blood (Petruška *et al.*, 2012). As the mineral content of the diets in this study were kept constant between the treatments, it was possible that the significant difference that arose was due to an increased absorption of phosphorus from the blood to the bones of P600 birds. Another plausible reason was that the P600 additive improved the apparent ileal nutrient digestibility of phosphorus, as demonstrated by Amad *et al.* (2011) who tested the phytogenic additives, thyme and star anise, on broiler growth and nutrient digestibility in the small intestine.

As differences did not exist for all of the tested bone parameters when comparisons were made against the negative control, it was recommended that further research be conducted with additional measures, inclusion levels, etc. so as to better investigate the effects of VivoCare® on skeletal characteristics.

Table 5.9 Average (± standard error) fat free dry weight; ash, calcium and phosphorus content; and Ca:P ratio of tibia bones from broilers slaughtered at 35 days of age that received different concentrations of the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Fat free dry bone weight (g)	Ash % of FFDBW[1]	Ca as % of bone ash	P as % of bone ash	Ca:P of bone ash
P500	4.72 ± 0.06	50.34 ± 0.51	44.58 ± 2.90	18.60^{ab} ± 0.29	2.39 ± 0.13
P600	4.68 ± 0.21	48.81 ± 0.38	44.51 ± 2.84	19.84^{a} ± 0.39	2.25 ± 0.17
P800	4.56 ± 0.31	47.39 ± 1.24	40.06 ± 2.43	18.47^{ab} ± 0.37	2.17 ± 0.12
POS	5.10 ± 0.15	47.85 ± 0.77	43.75 ± 3.77	17.79^{b} ± 0.38	2.45 ± 0.19
NEG	4.89 ± 0.27	47.32 ± 0.38	42.93 ± 2.76	18.32^{ab} ± 0.36	2.30 ± 0.17
***P* value**	0.469	0.039[2]	0.812	**0.008**	0.726

^{a,b} Means within columns with different superscripts differ significantly (P <0.05); [1]Fat free dry bone weight; [2]Accepted as statistically insignificant after performing *post-hoc* comparison tests; P500: 500 mg of VivoCare® per kg of feed; P600: 600 mg of VivoCare® per kg of feed; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

5.4. Conclusion

In conclusion, results from this study showed that, in comparison to the negative control, the use of the phytogenic additive, VivoCare®, had no significant overall effect on the carcass quality or skeletal parameters in this study. Differences that did exist yielded inconclusive results and it was only possible to speculate on the possible causes of these differences. Correlations from this study indicated that heavier bones could be associated with longer, thicker and stronger measurements, however, bones that were thicker were not also necessarily stronger. Further research is recommended to investigate this phytogenic additive under different circumstances (i.e. under the influence of an intentional stressor) or at different inclusion levels; due to the overall lack of differences between the treatment groups and the negative control, and the inconsistency of the observed differences seen in this study. It is also recommended that additional parameters be included in future studies, such as; carcass fat content, bone density, mineral digestibility, and cortical and trabecular bone thickness.

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Chapter 6

The effect of a phytogenic feed additive on the Descriptive Sensory Analysis of breast fillets of broiler chickens

Abstract

The purpose of this study was to investigate the effect of the phytogenic feed additive, VivoCare®, on the sensory profile (aroma, flavour and texture attributes) and physical characteristics (muscle pH, thaw loss and cooking loss) of broiler breast meat fillets. VivoCare® is produced by Beonics Feed Supplements (Pty) Ltd, and contains bio-active components originally identified from propolis. The fillets were obtained from a trial with mixed sex, Cobb 500 broiler chickens, fed three experimental diets, namely: a positive control (POS), a negative control (NEG), and a diet containing 800 mg of VivoCare® per kg of feed (P800). The P800 group did not differ significantly from the POS or NEG group for any of the sensory or physical attributes that were tested. The only significant difference observed, was whereby NEG had a more intense wet-feather/sweaty/barnyard aroma than POS. The PCA bi-plot and correlation coefficients indicated that the wet-feather attributes tended to associate positively with thaw loss and initial juiciness, which may have been due to a change in the meat membrane integrity and degree of volatile release, caused by oxidative processes. The antioxidant capabilities of POS could have induced meat tissue stability, which could have been the reason for the less prominent wet-feather aroma and associated decrease in thaw loss for this treatment. Further research is recommended, due to the lack of differences and experimental evidence to support the speculations raised in this study.

Keywords: 4-ethylphenol, breast pH, caffeoylquinic acid, cooking loss, correlation coefficient, principle component analysis, prodelphinidin, propolis, thaw loss, wet-feather/sweaty/barnyard

6.1. Introduction

In the past two decades, the preference for chicken breast meat has evolved accompanying the increase in demand for further processed meat products (Fletcher, 2002; Petracci *et al.*, 2015). One of the reasons for the popularity of breast meat is due to its healthy nutritional profile, i.e. in comparison to other meats, it has a lower fat content and contains a higher proportion of unsaturated fatty acids (Fletcher, 2002; Petracci *et al.*, 2015). Breast meat is also favoured due to its tenderness and mild flavour which makes it suitable for a wide variety of processed meat products (Petracci *et al.*, 2013). For home-cooking, it is a quick and easy meat to prepare and these qualities appeal to modern societies, where less and less time is seemingly spent on meal preparation (Petracci *et al.*, 2013).

Sensory analysis is one of the oldest forms of quality control and is still actively used today to understand and predict consumer preference, which in turn promotes competitiveness in the market place (Gridgeman, 1967; Haščík *et al.*, 2011). The three categories of sensory attributes include aroma, flavour and texture, which,

accompanied by the physical characteristics: meat pH, thaw loss and cooking loss; can all influence consumer satisfaction and the likeliness of repurchase (Fletcher, 2002; Pieterse *et al.*, 2014).

Muscle pH, in particular, has a prominent effect on meat quality. If meat pH is too acidic, i.e. 5.8 or lower, proteins reach their isoelectric point which reduces their water binding capacity, subsequently increasing thaw and cooking losses and potentially decreasing tenderness (Fletcher, 1999, 2002; Petracci *et al.*, 2015). On the other hand, meat with pH values exceeding 6, have shown increased rates of microbial spoilage which consequently impairs the meat appearance, aroma and flavour and also reduces shelf-life (Allen *et al.*, 1997; Petracci *et al.*, 2015).

With the ban of antimicrobials as growth promoters (AGPs) in animal feeds by the European Union in 2006 (Dibner & Richards, 2005; European Commission, 2005), much attention has focused on finding natural alternatives to replace AGPs. Phytogenic feed additives are plant-derived products which have been actively researched as plausible alternatives, due to their ability to exert positive effects on livestock health and performance (Windisch *et al.*, 2008). Studies have shown that the use of certain phytogenic additives in broiler feeds could somewhat enhance the sensory profile of meat. Some phytogenic examples that had this effect in broiler meat include: propolis (Haščík *et al.*, 2011), garlic and oregano essential oils (Kirkpinar *et al.*, 2014), garlic bulb and husk powder (Kim *et al.*, 2009), and dried milfoil and St. John's-wort herbage (Fritz *et al.*, 1993).

Propolis, a prime example of a phytogenic, is a complex, resinous product produced and used by honeybees from collected plant exudates; to protect themselves, their hive and their larvae from harmful micro-organisms (Banskota *et al.*, 2001). Propolis consists of numerous flavonoids and phenolic compounds, which are well known for their antioxidant and oxygen-derived free radical scavenging capabilities (Pascual *et al.*, 1994; Viuda-Martos *et al.*, 2008). Dietary supplementation with propolis can therefore potentially delay oxidative deterioration of meat and lipids (particularly polyunsaturated fatty acids), which is a process responsible for the development of rancid odours and flavours in meat, and the subsequent reduction in shelf-life (Wood & Enser, 1997; Antony *et al.*, 2006; Viuda-Martos *et al.*, 2008).

The purpose of this study was to investigate the effect of the phytogenic feed additive, VivoCare®, on the sensory profile (aroma, flavour and texture attributes) and physical characteristics (muscle pH, thaw loss and cooking loss) of broiler breast meat fillets. The Descriptive Sensory Analysis (DSA) technique was used to evaluate the sensory attributes. Statistical correlation tests and a Principle Component Analysis (PCA) were also conducted to assess potential relationships amongst parameters and the treatment groups. The trial was conducted with both a positive (the commercially used and available AGP, Zinc Bacitracin) and a negative control (AGP-free diet), and breast fillets from these birds were used to make final comparisons. It was hypothesised that the use of this phytogenic additive had no negative effects on the sensory profile of the broiler breast fillets.

6.2. Materials and Methods

6.2.1. Experimental animals and treatment diets

Ethical clearance was obtained from the Animal Ethics Committee of Stellenbosch University prior to the commencement of the trial; reference number SU-ACUD16-00018. A total of 330 day-old, mixed-sex, Cobb 500 broiler chicks were obtained from a commercial hatchery (County Fair, Hatchery 5, Klapmuts, Western Cape, South Africa). Chicks had been vaccinated against Newcastle Disease and Infective Bronchitis. The trial was conducted in a temperature controlled environment with artificial lighting at the Poultry Section of the Mariendahl Experimental Farm (Stellenbosch University). The temperature, ventilation and lighting of the house was set and controlled according to the COBB Broiler Management Guide (2013).

The 35 day trial consisted of five treatments replicated six times and was set out in a completely randomized manner. There were thus a total of 30 wire cages, each containing 11 chicks that were allocated at random. The cages were 0.9 m by 0.6 m in size and each contained a tube feeder and two nipple drinkers. Chicks were supplied feed and water *ad libitum* throughout the trial.

Chicks were fed a three phase diet consisting of a starter, grower and finisher. These diets were formulated according Cobb 500 requirements (Cobb 500 Broiler Performance and Nutrition Supplement, Cobb-Vantress Inc., Siloam Springs, AR), and the ingredients and nutrient composition of each phase can be seen in Table 3***.1*** of Chapter 3. Diets were allocated so that each chick received 900 g of starter (consumed over ± 18 days), 1200 g grower (consumed over ± 7 days), and then finisher up until the day of slaughter (consumed over ± 10 days). The five experimental diets consisted of a positive control containing the AGP Zinc Bacitracin (POS), a negative control (NEG), and three test diets which had inclusion levels of 500, 600 and 800 mg of VivoCare® per kg of feed (P500, P600 and P800). The VivoCare® product was produced by Beonics Feed Supplements (Pty) Ltd, after studying signal molecules and gene expression in different types of propolis, and it contains caffeoylquinic acids and prodelphinidin bioflavonoids as the main bio-active components.

For the DSA, breasts were only tested from treatments P800, POS and NEG. In line with the hypothesis being tested, it was assumed that when considering the same test product, the treatment with the highest inclusion level would have the most profound negative effect on the sensory profile, if any. It was for this reason that P500 and P600 were excluded from the DSA, and diet P800 was solely used to represent the effect of this phytogenic additive on the sensory profile. A description of the three experimental treatment diets can be seen in Table 6.1.

Table 6.1 A description of the three experimental treatments used throughout the trial

Treatment	Inclusion	Description
P800	800 mg/kg inclusion	VivoCare® (Beonics Feed Supplements (Pty) Ltd)
POS	150 mg/kg inclusion	Positive Control (AGP[1] - Zinc Bacitracin)
NEG	No AGP[1]	Negative control

[1]Antimicrobial growth promoter: Zinc Bacitracin

6.2.2. Sample preparation

Sensory analysis was performed on the breast fillets of broiler chickens from three treatment groups that were each replicated six times. Two representative birds from each cage (replication) were randomly selected; one for the sensory training session and one for the sensory testing session. A total of 36 birds were thus used (3 treatments x 6 replications x 2 session types). Both of the whole, intact, breast portions from each bird were removed, vacuum packed and frozen on the day of slaughter and were then thawed in a refrigerator at 4°C for 24 h prior to the sensory test. The breast portions were deboned and the left and right fillet (*M. pectolaris major*) of each bird were placed together in an oven bag (Glad®). The bagged fillets were placed on a stainless steel grid, which in turn was placed atop an oven roasting tray. The point of a thermocouple probe was positioned in the centre of one of the two fillets (American Meat Science Association, 2015) and was then connected to a handheld digital temperature monitor (Hanna Instruments, South Africa). The oven bags were subsequently closed with plastic ties and the trays with the fillets were put in an industrial oven (Hobart, France) which had been preheated to 160°C (American Meat Science Association, 2015). Once the core temperature had reached 75°C, the cooked, unseasoned fillets were removed from the oven and allowed to cool for 15 min. Both fillets from each bird were then cut into 1 x 1 x 1 cm cubes, selecting the inner, more uniformly cooked portions of the fillet where possible. Each cube was then loosely wrapped in aluminium foil and three (two for the training session) of the same sample cubes then placed into a glass ramekin for each of the judges. During the testing sessions, each ramekin represented a certain treatment, and each treatment was assigned a randomized 3-digit code. The ramekins were placed once again in the oven for 10 min, which had been preheated to 70°C. They were then removed and immediately placed in water baths (SMC, South Africa) (70°C) on top of porcelain cups, half filled with water, to keep the temperature constant. The panel then evaluated the samples from the water baths.

6.2.3. Descriptive sensory analysis

Descriptive sensory analysis (DSA) was performed on the three treatment groups during six consecutive testing sessions. The sensory panel consisted of 13 judges, all of whom had had previous experience with meat sensory analysis. The panel underwent six training sessions prior to testing, with the purpose of attribute generation and the calibration of each panellist's sensory perception. The training sessions had an additional 10 meat reference standards in conjunction with the five treatment samples, as described in Table 6.2. These

reference meats were used to assist in the recognition and scoring of specific attributes associated with aroma, flavour and texture when scoring the different treatment samples. Reference meat standards included: commercial chicken fillets, free range chicken fillets, commercial chicken thighs, overdone chicken fillets cooked to an internal temperature of 85°C, pre-cooked rotisserie chicken fillets, chicken livers, duck breast fillets, ostrich fillet, beef fillet and beef shin. Panellists were trained according to the guidelines for sensory analysis of meat, specified by the American Meat Science Association (2015) and followed the generic technique described by Lawless and Heymann (2010) for descriptive sensory analysis. During a single training session, each panellist received two cubes of each meat sample. At the end of the sixth training period, panellists decided on 17 sensory attributes that would be used to score the treatment samples during the testing phase. The chosen attributes and their corresponding description and scale can be seen in Table 6.3.

The test re-test methodology was followed for the DSA. Thirteen individual tasting stations, each with their own computer, were prepared in a temperature controlled room (21°C). The software programme Compusense® five (Compusense, Guelph, Canada) was used for the testing sessions to evaluate the decided attributes for each sample. Scoring was indicated on an unstructured line scale ranging from zero (extremely low intensity) to 100 (extremely high intensity) (American Meat Science Association, 2015). Each panellist received the three test samples in a completely random order; accompanied by distilled water (21°C), apple quarters and water biscuits (Carr, UK) as palate cleansers.

Table 6.2 Reference meats and the attributes they are associated with

Reference meat	**Attributes**
Chicken breast fillet – normal	Chicken-like
Chicken breast fillet – free range	Chicken-like
Chicken thigh	Fatty
Chicken breast fillet – overcooked	Dry, mealy
Rotisserie chicken breast fillet	Roasted chicken
Chicken liver	Livery, metallic
Duck breast fillet	Sweet, oil, grainy
Ostrich fillet	Metallic
Beef shin	Tough
Beef fillet	Tender

Table 6.3 Description and scale of each sensory attribute used for the descriptive sensory analysis

	Sensory Attribute	Description	Scale
Aroma	Overall meat aroma	Overall intensity of the meat aroma in the first few sniffs as soon as the foil is removed	0 = Extremely mild 100 = Extremely intense
	Chicken-like aroma	Aroma associated with typical cooked chicken as soon as the foil is removed	0 = Extremely mild 100 = Extremely intense
	Roasted aroma	Aroma associated with roasted chicken as soon as the foil is removed	0 = Extremely mild 100 = Extremely intense
	Sweet aroma	Intensity of a sweet, slightly oily aroma, associated with grain or the Maillard reaction, as soon as the foil is removed	0 = Extremely mild 100 = Extremely intense
	Fatty aroma	The intensity of a fatty aroma associated with roasted chicken, as soon as the foil is removed	0 = Extremely mild 100 = Extremely intense
	Wet-feather/ sweaty/barnyard aroma[1]	Aroma associated with a wet chicken coop; combination of feed, manure, mouldy hay, and wet poultry feathers	0 = Extremely mild 100 = Extremely intense
Flavour	Overall meat flavour	Overall intensity of the meat flavour in the first few chews prior to swallowing	0 = Extremely mild 100 = Extremely intense
	Chicken-like flavour	Flavour associated with typical cooked chicken prior to swallowing	0 = Extremely mild 100 = Extremely intense
	Roasted flavour	The flavour associated with roasted chicken prior to swallowing	0 = Extremely mild 100 = Extremely intense
	Sweet flavour	Intensity of a sweet slightly oily and grainy flavour associated with a grain-like or starchy character, or the Maillard reaction	0 = Extremely mild 100 = Extremely intense
	Fatty flavour	Flavour associated with cooked/roasted chicken fat prior to swallowing	0 = Extremely mild 100 = Extremely intense
	Wet-feather/ sweaty/barnyard flavour[2]	Flavour associated with a wet chicken coop; combination of feed, manure, mouldy hay, and wet poultry feathers	0 = Extremely mild 100 = Extremely intense
Texture	Mealiness	Extremely fine, powdery texture; disintegration of muscle fibre into very small particles that are retained on the palate	0 = Not mealy 100 = Extremely mealy
	Initial juiciness	Amount of fluid exuded when pressed between thumb and forefinger	0 = Extremely dry 100 = Extremely juicy
	Sustained juiciness	Impression formed after first 5 chews using molar teeth	0 = Extremely dry 100 = Extremely juicy
	Tenderness	Impression of tenderness after the first 5 chews using molar teeth	0 = Extremely tough 100 = Extremely tender
	Residue	Residual tissue remaining after the first 10 chews	0 = None 100 = Abundant

[1]Will be further referred to as wet-feather aroma
[2]Will be further referred to as wet-feather flavour

6.2.4. Physical attributes

Prior to the cooking process, once the chicken breasts had thawed for 24 h, pH measurements were taken for the three meat samples before each of the six testing sessions. Measurements were taken using a portable Crison pH 25, handheld portable pH meter (Lasec (Pty) Ltd, South Africa), with an automatic temperature adjuster. The pH meter was calibrated prior to each session with the standard buffers (pH 4.0 and pH 7.0)

supplied by the manufacturer. When measurements were taken, the probe of the pH meter was carefully inserted into the meat at the centre of the breast and positioned so as to obtain a stable and accurate reading.

After slaughter, when the carcass was divided into portions, the breast portions for the DSA were removed and weighed using a Mettler PC 4400 scale (Mettler-Toledo, Switzerland). They were then vacuum-packed and frozen (–18°C) for approximately five weeks. Prior to each of the six DSA testing sessions, the breast portions were thawed at 4°C for 24 h, blotted dry with blotting paper and weighed. The thaw loss was calculated using Equation 6.1, and represented the amount of moisture lost by an entire breast portion (fillet, skin and bone) after being vacuum-packed, frozen and thawed.

Cooking losses were determined for the breast fillets (*M. pectolaris major*) after thawing and once the breast portions had been deboned. This was done according to methods described by AMSA (2015) and calculations were performed using Equation 6.2. Cooking losses were recorded for the three meat samples at each of the six sensory testing sessions.

Equation 6.1: $$\text{Thaw Loss (\%)} = \frac{\text{Breast weight after thawing (g)}}{\text{Breast weight prior to freezing (g)}} \times 100$$

Equation 6.2: $$\text{Cooking loss (\%)} = \frac{\text{Fillet weight after cooking (g)}}{\text{Fillet weight before cooking (g)}} \times 100$$

6.2.5. Statistical analysis

A trained panel of 13 judges evaluated the 17 different sensory attributes for each sample during each of the six testing sessions. PanelCheck Software (Version 1.3.2, http://www.panelcheck.com/) was used to monitor DSA panel performance. The collected sensory data was analysed using a test re-test Analysis of Variance (ANOVA). This was performed using SAS® statistical software (Statistical Analysis System 2006, Version 9.2, SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was conducted to test for the non-normality of residuals (Shapiro & Wilk, 1965). In the event of non-normality (P <0.05), observations with residuals larger than the value three were regarded as outliers and subsequently removed from the data set. If differences (P <0.05) existed after the final ANOVA had been conducted, multiple comparison *post-hoc* tests were performed using Bonferroni (Dunn) t Tests.

In order to visually clarify and indicate possible relationships between the treatment groups, and sensory and physical attributes, a Principal Component Analysis (PCA) bi-plot was constructed (Næs *et al.*, 2011). The statistical software used for this multivariate analysis was XLSTAT (Version 2016, Addinsoft, New York, USA). Pearson's correlation coefficients (r) were also used to assess the correlations between the different sensory and physical attributes (Snedecor & Cochran, 1989). Correlations were significant when P <0.05 and the correlation measures were strongest when they approached the values 1 or -1. To interpret the strength of the r values, the guide as given in Table 6.4 was used (Evans, 1996).

Table 6.4 The guide used to verbally describe the strength of Pearson's correlation coefficient, *r* (Evans, 1996)

If the absolute value of *r* is in this range:	The strength of *r* can be verbally described as:
0.00 – 0.19	Very weak
0.20 – 0.39	Weak
0.40 – 0.59	Moderate
0.60 – 0.79	Strong
0.80 – 1.00	Very strong

r : Pearson's correlation coefficient

6.3. Results and Discussion

6.3.1. Sensory attributes

The average scores for the aroma, flavour and texture attributes obtained from the DSA can be seen in Table 6.5, Table 6.6 and Table 6.7, respectively. The more prominent attributes, with values between 60 and 80 on the line scale, included: overall meat and chicken-like aroma and flavour, as well as tenderness. Mealiness, initial juiciness and sustained juiciness were moderately prominent attributes with values ranging between 25 and 40; while the low intensity attributes included: residue, along with roasted, sweet, fatty, and wet-feather, aroma and flavour, with values ranging from 10 to 20.

It was seen that the only sensory attribute to have differences (P <0.05) was that of the wet-feather aroma attribute. The results indicated that judges perceived a significantly stronger wet-feather aroma in the negative control meat as opposed to the positive control meat. It was noted, however, that this particular aroma was not intense or overpowering for any of the meat samples, but instead mild or slight, as seen by the low average scores for this attribute (i.e. falling between 12 and 16 on the line scale). Nevertheless, this aroma was distinct enough to elicit a significant difference between treatment samples.

Very little research exists on the wet-feather attribute in poultry meat. This attribute can, however, be considered unpleasant and undesirable, especially at higher intensities, and can therefore be referred to as an off-aroma/flavour attribute. Poultry-related publications which have mentioned the attribute, only tested it for flavour and did not show any significant differences between groups. They also, similarly, indicated that this attribute had lower scores on the intensity line scale; more closely resembling a mild or slight taint, rather than a prominent and overpowering off-flavour. (Zhuang *et al.*, 2007; Zhuang & Savage, 2010; Zhuang & Bowker, 2014).

There is currently no literature suggesting the reason for the existence of this wet-feather attribute in chicken meat. An off-odour with similar descriptors has, however, been found in red wine. This phenolic off-odour is commonly known as the "Brett character" and terms that have been used to describe this taint include: horse-sweat, animal and barnyard (Chatonnet *et al.*, 1992, 1995; Suárez *et al.*, 2007; Lisanti *et al.*, 2017). It has been said that the cause of this taint is due to the volatile organic compound (VOC) 4-ethylphenol, which is

predominantly produced in wine by the spoilage yeast *Brettanomyces/Dekkera* (Chatonnet *et al.*, 1992, 1995; Dias *et al.*, 2003). Heterocyclic compounds have been shown to have low odour threshold values and it is for this reason that, even at relatively low concentrations, they can have a significant effect on aroma (Mottram, 1998; Jayasena *et al.*, 2013).

With regards to VOCs in livestock, it was indicated that 4-ethylphenol was a prominent VOC in chicken manure (Huang *et al.*, 2007; Shah *et al.*, 2016). It was suggested that dietary protein could be a possible pre-cursor for this phenolic compound, as the amino acid tyrosine could potentially be degraded to 4-ethylphenol and other phenols in the digestive tract of mammals (Spoelstra, 1980; Ha & Lindsay, 1991; Lane & Fraser, 1999). This compound has also been found in markedly higher concentrations in the fat and urine of grain-fed beef cattle in comparison to those that were grass-fed (Lane & Fraser, 1999).

VOCs, both desirable and undesirable, have been shown to originate from mild thermal oxidative changes of lipids, especially unsaturated fatty acids, in cooked meats (Shahidi, 2002; Jayasena *et al.*, 2013). We can therefore speculate that the wet-feather aroma originated due to the grain-based diets in this trial, and that the taint was more prominent in the negative control meat due to potentially higher concentrations of 4-ethylphenol in the meat or fat of these birds. The AGP treatment (POS), which did not significantly differ from the phytogenic treatment (P800) for this attribute, was more successful in reducing this off-aroma taint in the meat and could be as a result of some unknown process potentially related to reduced tyrosine degradation or reduced lipid oxidation. These are all, however, speculations and further research is strongly recommended, as these conjectures require much analytical support and research-based evidence.

Table 6.5 Average (± standard error) scores for the aroma attributes evaluated for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Overall meat aroma	Chicken-like aroma	Roasted aroma	Sweet aroma	Fatty aroma	Wet-feather aroma
P800	63.91 ± 0.31	63.02 ± 0.69	12.50 ± 0.39	11.22 ± 0.22	10.96 ± 0.08	14.16^{ab} ± 0.83
POS	62.95 ± 1.09	63.19 ± 0.95	13.32 ± 0.77	12.10 ± 0.46	10.97 ± 0.09	12.95^{b} ± 0.69
NEG	64.11 ± 0.75	63.45 ± 0.60	13.34 ± 0.59	11.46 ± 0.38	10.89 ± 0.07	15.69^{a} ± 0.63
***P* value**	0.552	0.924	0.544	0.251	0.744	**0.049**

[a,b] Means within columns with different superscripts differ significantly (P <0.05); P800: 800 mg of the phytogenic additive per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 6.6 Average (± standard error) scores for the flavour attributes evaluated for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Overall meat flavour	Chicken-like flavour	Roasted flavour	Sweet flavour	Fatty flavour	Wet-feather flavour
P800	64.60 ± 0.28	63.27 ± 0.42	12.38 ± 0.52	12.36 ± 0.42	11.08 ± 0.12	13.45 ± 0.93
POS	63.78 ± 0.56	62.58 ± 0.46	11.91 ± 0.41	12.35 ± 0.56	11.12 ± 0.09	12.81 ± 0.47
NEG	64.71 ± 0.44	63.24 ± 0.50	12.99 ± 0.59	13.03 ± 0.34	11.19 ± 0.09	13.08 ± 0.70
***P* value**	0.295	0.506	0.354	0.486	0.756	0.820

Differences are significant when $P < 0.05$; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

Table 6.7 Average (± standard error) scores for the texture attributes evaluated for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Mealiness	Initial juiciness	Sustained juiciness	Tenderness	Residue
P800	31.05 ± 1.12	37.92 ± 1.66	36.17 ± 0.97	77.89 ± 1.77	12.30 ± 0.79
POS	28.86 ± 0.64	39.33 ± 1.34	37.92 ± 0.97	76.59 ± 1.25	12.76 ± 0.46
NEG	30.84 ± 1.04	38.92 ± 0.92	35.46 ± 1.42	77.52 ± 1.56	12.75 ± 0.53
***P* value**	0.236	0.752	0.319	0.830	0.832

Differences are significant when $P < 0.05$; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

6.3.2. Physical attributes

Table 6.8 presents the average values obtained for the physical attributes: breast pH, thaw loss (%) and cooking loss (%), for broilers receiving the phytogenic feed additive, VivoCare®, versus a positive and negative control diet. It was seen that there were no differences ($P > 0.05$) between the three experimental diets for any of these attributes. The lack of significant differences suggested that no obvious links could be made between the physical attributes and the observed difference noted for the wet-feather aroma attribute. The values obtained in this study for these physical attributes were in line with those found for standard commercial broiler fillets from other studies (Zhuang & Savage, 2010; Geldenhuys *et al.*, 2014).

Table 6.8 Average (± standard error) pH, thaw loss and cooking loss values obtained for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control

Treatment	Breast pH	Thaw loss (%)	Cooking loss (%)
P800	5.84 ± 0.03	4.46 ± 0.31	22.57 ± 2.06
POS	5.83 ± 0.02	4.18 ± 0.49	23.44 ± 0.77
NEG	5.83 ± 0.02	5.21 ± 0.32	23.53 ± 0.80
***P* value**	0.894	0.175	0.857

Differences are significant when $P < 0.05$; P800: 800 mg of VivoCare® per kg of feed; POS: Positive control containing an antimicrobial growth promoter (Zinc Bacitracin); NEG: Negative control containing no Zinc Bacitracin or feed additives

6.3.3. Principle Component Analysis and Pearson's correlation coefficients

A Principal Component Analysis (PCA) bi-plot, illustrated in Figure 6.1, was constructed to aid in making visual associations between the attributes and the three experimental treatment groups. This bi-plot only explained 45.82% of the total variation, nevertheless, this was still high with regards to sensory analysis, as there are numerous factors which could have possibly influenced the sensory profile. From the PCA plot it can be seen that the replications of each treatment group did not form obvious and distinctly separated clusters, but instead were quite widely spread and seen to overlap. The overlaps in this case would be due to the lack of significant differences between the treatments for the different attributes. This absence of differences when many variables are being compared, has also been known to induce artificial separation which would explain the wide and almost random spread of some of the treatment replications.

Pearson's correlation coefficients (*r*) and their corresponding degrees of significance are also given for the physical, texture, flavour and aroma attributes in Table 6.9 and Table 6.10, to aid in statistically evaluating potential associations illustrated by the PCA plot. Significant correlations were seen to range from 0.469 to 0.781 (absolute values) and will be further described as positive or negative; with a moderate (|0.40 to 0.59|[1]) or strong (|0.60 to 0.79|) correlation as described by Evans (1996) and as illustrated in Table 6.4.

6.3.3.1. "Commercial chicken-like" attributes and associations

It was noted that there were many significant positive correlations between the attributes: chicken-like, roasted, fatty, sweet, and overall, aroma and flavour. These attributes are all typical of commercially available chicken meat and seemed to be all positively correlated in one way or another (Jayasena *et al.*, 2013). Prominence in one attribute thus tended to reflect prominence in the others. This effect was confirmed by the PCA plot, where all of these attributes were grouped together on the right hand side of the F1 axis.

It was also noted that of the three treatment groups, the positive control's replications grouped closest to these "commercial chicken-like" attributes. The negative control, on the other hand, tended to group on the opposite

[1] Absolute value is indicated by ||, which in this case implies a value between 0.40 and 0.59; or -0.40 and -0.59

or negative side of the F1 axis associating more with the wet-feather aroma and flavour. The P800 group, which did not differ significantly from either of the control groups with regards to the wet-feather attribute, can be seen to spread randomly across the PCA plot not associating particularly with either side of the F1 axis, which visually confirmed the lack of obvious differences and associations for this treatment group.

6.3.3.2. Wet-feather attribute associations

It was seen that according to Pearson's correlation coefficients, the wet-feather aroma attribute only correlated significantly with one other attribute, namely; thaw loss. This strong positive correlation (r =0.642) indicated that the wet-feather aroma was more intense for meat that had lost more moisture during thawing. Pre-slaughter stress, such as heat stress, has been shown to increase protease and lipase activation, as well as increase the production of reactive oxygen species (Petracci *et al.*, 2010, 2015). Both of these processes can reduce membrane integrity which in turn decreases the water holding capacity (WHC) of meat and subsequently increases drip and thawing losses (Van Laack *et al.*, 2000; Petracci *et al.*, 2015). These processes also increase the rate of post mortem lipid and protein oxidation, which may contribute to the production of volatiles and off-odours (Nam & Ahn, 2003), such as the slight, yet discernible, wet-feather aroma found in this study. The antioxidant capabilities of the positive control and P800 group could have induced *in vivo* and post mortem tissue stability and this could potentially be the reason for the less prominent wet-feather aroma in these treatment groups, as well as for the noted correlation with thaw loss (Ajuyah *et al.*, 1993; Patterson & Stevenson, 1995; Nam & Ahn, 2003).

The wet-feather flavour attribute had a moderate positive correlation with wet-feather aroma (r =0.569), which was confirmed by the close proximity of these two attributes to one another, in the top left quadrant of the PCA plot. The wet-feather flavour attribute also had moderate negative correlations with fatty aroma (r = -0.512), and roasted aroma (r = -0.475) and flavour (r = -0.480). These results confirmed a statement by Jayasena *et al.* (2013) who claimed that off-flavours can be accompanied by a parallel reduction in chicken "meaty" flavours and aromas. The wet-feather flavour attribute, also had a moderate positive correlation to thaw loss (r =0.535), again suggesting that this off-taint was more intense for meat that had lost more moisture during thawing; which, as mentioned before, could be due to reduced membrane integrity and increased rates of meat oxidation (Nam & Ahn, 2003). Lastly, wet-feather flavour had a moderate positive correlation with initial juiciness (r =0.480). The attribute initial juiciness has been used in organoleptic analyses to represent the capacity of meat to exude its constitutive water (Dryden & Maechello, 1970). It was possible that a decrease in membrane integrity accompanying an increased rate of muscle oxidation, gave rise to the simultaneous release of water and wet-feather volatiles, which combined to form a wet-feather-type broth (Sheridan *et al.*, 2003; Jayasena *et al.*, 2013). This could have been a possible reason why when initial juiciness increased so did the intensity of the wet-feather flavour.

These associations can be viewed on the PCA plot where the wet-feather attributes, thaw loss and initial juiciness all appear to associate closely in the top left quadrant, while the so-called "meaty" attributes (fatty aroma, and roasted aroma and flavour) could be found diagonally opposite confirming the negative correlations.

6.3.3.3. Physical attribute associations

For the physical attributes, it was seen that breast meat pH had moderate positive correlations with tenderness (r =0.487), roasted flavour (r =0.510), and chicken-like aroma (r =0.483) and flavour (r =0.542). The meat pH also had moderate negative correlations with residue (r = -0.506) and thaw loss (r = -0.554). It could thus be said that if meat pH decreased and became more acidic, thaw loss would increase and meat would became less tender, with more residue and less intense roasted and chicken-like attributes. Cooking loss was also seen to have a moderate negative correlation with tenderness (r = -0.480) and a moderate positive correlation with residue (r =0.469).

Previous studies have shown that lower meat pH values can be associated with decreased WHC and a higher drip loss as the isoelectric point of proteins is reached (Petracci *et al.*, 2015), and this would explain the negative correlation with thaw loss as seen in this study. The loss of moisture which occurred during the thawing and cooking processes could have indicated that there was less water available to hydrate the muscle fibres, which may have increased the number of fibres per surface area and could have then been perceived as toughness by the sensory panellists i.e. meat that was less tender with more residue (Hughes *et al.*, 2014; Zhuang & Bowker, 2014). It has been said that chicken meat can lose its characteristic flavour through the process of water extraction (Koehler & Jacobson, 1967; Hopkins *et al.*, 2006), and this may be the reason for the correlations observed between pH and the “commercial chicken-like” attributes; as the pH and WHC decrease, so did the meats ability to retain its characteristic chicken-like flavour and aroma.

These associations for the physical attributes can be seen on the PCA plot where pH, tenderness, roasted flavour, and chicken-like aroma and flavour, are grouped together in the top right quadrant on the opposite side of the F1 axis from residue and thaw loss. Cooking loss and residue are also grouped closely and opposite to tenderness.

6.3.3.4. Texture attribute associations

Tenderness and residue were seen to have a strong negative correlation (r = -0.781) which can also be seen by their diagonally opposing positions on the PCA plot and which was similarly reported by Geldenhuys *et al.* (2014) for poultry meat. Interestingly, mealiness showed moderate positive correlations to overall meat flavour (r =0.493), chicken-like flavour (r =0.539) and sweet flavour (r =0.488). In contrast, initial juiciness showed a moderate negative correlation to chicken-like flavour (r = -0.529); while sustained juiciness had strong and moderate negative correlations with roasted flavour (r = -0.635), and chicken-like (r = -0.554) and sweet flavour (r = -0.527), respectively.

With regards to the PCA plot, mealiness was thus positioned to the right on the F1 axis, associating with the “commercial chicken-like” flavours; while initial and sustained juiciness were found to be on the left hand side of the F1 axis, associating with the wet-feather flavour. Joubert *et al.* (2013) showed similar associations and positioning’s for mealiness, initial and sustained juiciness on a PCA bi-plot, when investigating sensory and physical attributes for commercial (intensively produced) versus free range broiler breast fillets. Joubert *et al.* (2013) indicated that the commercial control meat associated more strongly with mealiness and chicken-like

flavour, as did the positive control in the current study. In contrast, the free range meat in the study by Joubert *et al.* (2013), grouped with initial and sustained juiciness; while in this study, it was the negative control, along with the wet-feather attributes, which seemingly took up this position. Based on these similarities, if further organoleptic studies with *VivoCare*® are conducted, it may be useful to include free range breast meat as another comparative control.

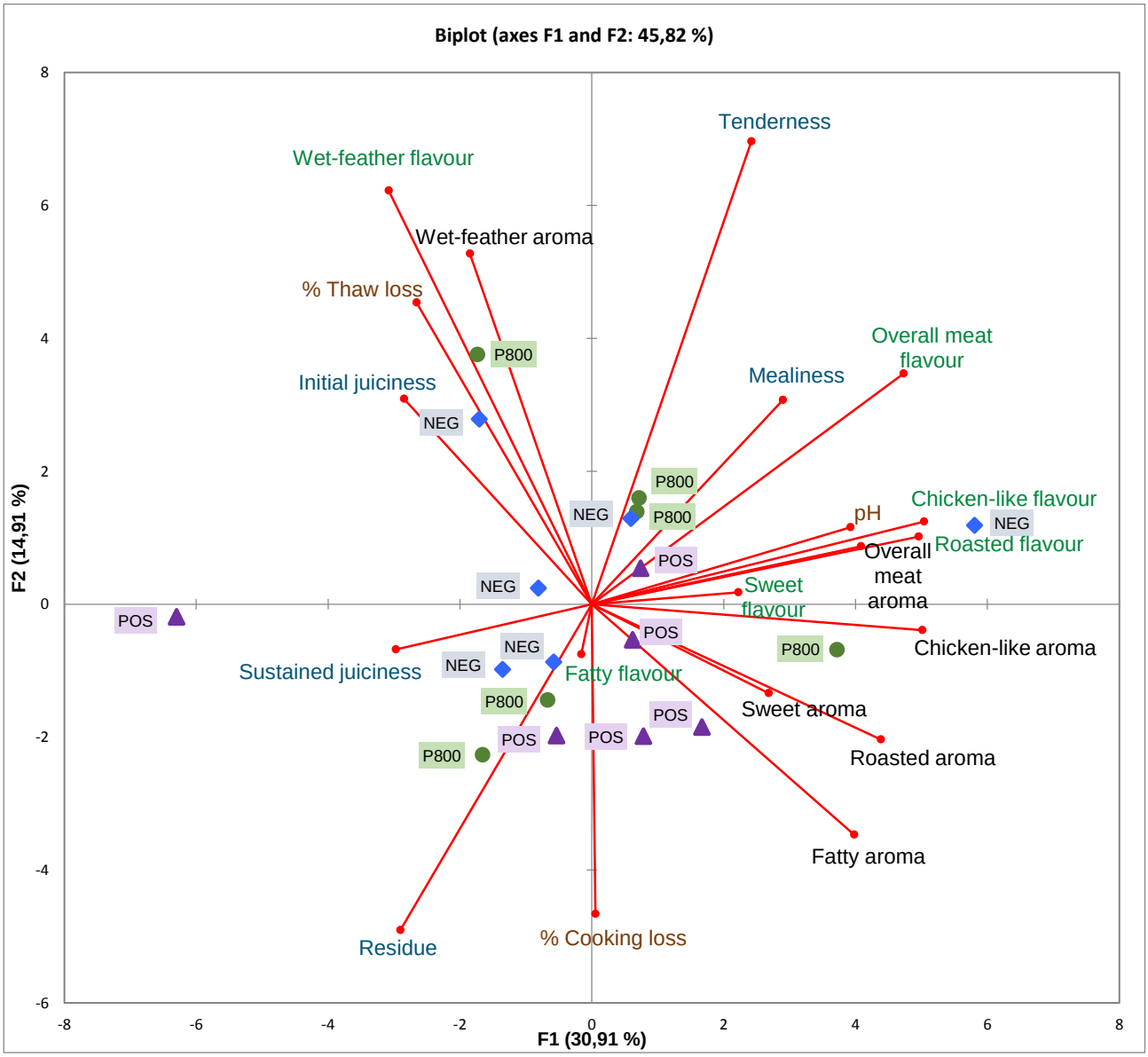

Figure 6.1 Principle Component Analysis of sensory attributes for breast meat from broilers grown from hatch to 35 days receiving three different treatment diets (P800 - 800 mg of VivoCare® per kg of feed; POS - Positive control containing the antimicrobial growth promoter, Zinc Bacitracin; NEG - Negative control containing no Zinc Bacitracin or feed additives)

Table 6.9 Pearson's correlation coefficients between aroma, flavour, texture and physical attributes evaluated for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control

	Physical attributes			Texture				
	Cooking loss	**Thaw loss**	**pH**	**Residue**	**Tenderness**	**Sustained juiciness**	**Initial juiciness**	**Mealiness**
Aroma								
Overall meat aroma	- 0.037	- 0.005	0.302	- 0.149	0.147	- 0.170	- 0.189	0.278
Chicken-like	0.042	- 0.191	**0.483***	- 0.388	0.265	- 0.104	- 0.247	0.253
Roasted	- 0.051	- 0.392	0.245	- 0.324	0.090	- 0.056	- 0.295	0.172
Sweet	- 0.126	- 0.168	0.164	- 0.036	- 0.022	- 0.156	0.099	0.043
Fatty	0.197	- 0.178	0.127	0.042	- 0.096	- 0.146	- 0.356	0.236
Wet-feather	0.116	**0.642****	- 0.256	0.070	0.166	- 0.127	0.222	0.097
Flavour								
Overall meat flavour	- 0.027	- 0.026	0.378	- 0.465	**0.497***	- 0.376	- 0.262	**0.492***
Chicken-like	0.062	- 0.214	**0.542***	- 0.399	0.445	**- 0.554***	**- 0.529***	**0.539***
Roasted	- 0.087	- 0.270	**0.510***	- 0.405	0.368	**- 0.635****	- 0.352	0.413
Sweet	0.185	- 0.182	0.022	0.060	0.174	**- 0.527***	- 0.098	**0.488***
Fatty chicken	- 0.087	0.202	- 0.185	- 0.083	- 0.121	0.401	0.090	0.261
Wet-feather	- 0.290	**0.535***	- 0.214	- 0.101	0.322	0.243	**0.480***	- 0.087
Texture								
Mealiness	- 0.039	0.024	0.118	- 0.198	0.339	- 0.413	0.157	-
Initial juiciness	- 0.299	0.415	- 0.275	0.170	- 0.031	0.076	-	-
Sustained juiciness	- 0.202	0.187	- 0.386	- 0.017	- 0.041	-	-	-
Tenderness	**- 0.480***	0.076	**0.487***	**- 0.781*****	-	-	-	-
Residue	**0.469***	0.148	**- 0.506***	-	-	-	-	-
Physical attributes								
pH	- 0.061	**- 0.554***	-	-	-	-	-	-
Thaw loss	- 0.213	-	-	-	-	-	-	-

*, **, and *** indicate significance at P <0.05, P <0.01, and P <0.001, respectively

Table 6.10 Pearson's correlation coefficients between aroma and flavour, evaluated for breast meat from broilers grown from hatch to 35 days receiving the phytogenic additive, VivoCare®, versus a positive and negative control

	Flavour						Aroma				
	Wet-feather	**Fatty**	**Sweet**	**Roasted**	**Chicken-like**	**Overall meat flavour**	**Wet-feather**	**Fatty**	**Sweet**	**Roasted**	**Chicken-like**
Aroma											
Overall meat aroma	- 0.071	0.020	- 0.082	0.350	0.416	**0.762*** **	0.048	**0.623** **	**0.543***	**0.499***	**0.763*** **
Chicken-like	- 0.314	0.180	- 0.024	**0.484***	**0.516***	**0.649** **	- 0.269	**0.693** **	**0.530***	**0.750*** **	-
Roasted	**- 0.475***	0.147	0.231	**0.490***	0.312	**0.501***	- 0.412	0.431	**0.539***	-	-
Sweet	- 0.135	- 0.067	- 0.081	0.036	0.168	0.338	- 0.264	0.468	-	-	-
Fatty	**- 0.512***	0.174	0.068	0.375	**0.537***	0.367	- 0.366	-	-	-	-
Wet-feather	**0.569***	- 0.084	0.087	- 0.129	- 0.103	0.152	-	-	-	-	-
Flavour											
Overall meat flavour	- 0.034	- 0.191	0.278	**0.553***	**0.590** **	-	-	-	-	-	-
Chicken-like	- 0.326	0.088	0.316	**0.716*** **	-	-	-	-	-	-	-
Roasted	**- 0.480***	- 0.099	**0.570***	-	-	-	-	-	-	-	-
Sweet	- 0.387	- 0.287	-	-	-	-	-	-	-	-	-
Fatty	- 0.153	-	-	-	-	-	-	-	-	-	-

*, **, and *** indicate significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

6.4. Conclusion

The results of this study indicated that the phytogenic additive, VivoCare®, did not differ significantly from the positive or negative control for any of the sensory or physical attributes that were tested, and therefore did not have any negative effects on the sensory profile. In fact, the only significant difference that was observed in this study, was the significantly more intense wet-feather/sweaty/barnyard aroma that was perceived by judges for the negative control meat, in comparison to the positive control meat. It was speculated that this off-odour was as a result of a volatile organic compound (VOC), such as 4-ethylphenol, which may have been more prominent in the negative control due to oxidative processes. Oxidation may have also played a role in the positive associations between the wet-feather attributes and thaw loss and initial juiciness.

Additional research is recommended due to the lack of significant results found in this study. It may be worthwhile investigating other concentrations of the VivoCare® product in broiler diets, while also investigating effects under different conditions (i.e. in the presence of an intentional stressor). Further studies are also recommended to investigate the wet-feather attribute more thoroughly; the use of an electronic nose (e-nose) would be a helpful device for the identification of specific odour components. Other experimental tests and procedures which could analyse and compare VOCs and fatty acid concentrations, as well as, oxidative rates between the experimental meat groups, would also add great value to future studies.

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Chapter 7

General Conclusion

With the use of AGPs in animal production slowly being phased out due to the increased risk of untreatable AMR-related illnesses, accompanied by consumer preference for a healthier meat product (i.e. antimicrobial free); the search for potential alternatives has become an important field of study in the 21st century. As broiler meat is the predominant source of protein in South Africa, the identification and use of feasible alternatives will help to correct the negative impacts of AMR on public health, whilst also making South African poultry products more acceptable for export to countries with AGP bans.

This study was conducted with the assumption that in order for the phytogenic additive, VivoCare®, to have any potential viability as an AGP alternative in a commercial setting, it had to significantly out-perform the negative control, unless the positive and negative control were statistically similar. Significant overall differences were, however, absent in the production parameter trial; whereby results showed that the negative control, positive control and three VivoCare® test diets all performed equally well in terms of growth performance. A possible reason for the lack in differences could have been due to the birds being raised in an optimal environment that was reasonably stress- and pathogen-free. As it is known that AGPs do not promote growth in these optimal conditions, the absence of additional growth promoting effects from the VivoCare® fed birds in this study was no surprise. The overall lack of differences in feed intake also confirmed that the form and concentration of the tested additives had no negative effects on feed quality and palatability.

Results from the toxicity study showed that VivoCare® did not cause any significant gizzard erosion, even when inclusion levels were doubled. This confirmed that the product was non-toxic and safe to use as a feed additive. With regards to the other organ and intestinal parameters, significant overall differences were again absent between the VivoCare® and the control groups indicating similar gut health and immune status. It was noted that the negative control did seem to show slightly more evidence of exposure to immunological stress, however, differences were not prominent and other measures of gut health and immune response should be further investigated to better support these results.

The VivoCare® diets also had no significant overall effects on the carcass quality or skeletal parameters in this study. The few differences that did exist yielded inconclusive results and it was only possible to speculate on the possible causes of these differences. Results from correlation tests indicated that heavier bones could be associated with longer, thicker and stronger measurements, however, bones that were thicker were not also necessarily stronger.

Results for the final chapter also indicated a lack of significant differences between the VivoCare® group and the two controls for the sensory and physical attributes that were tested. The use of VivoCare® in broiler diets at 800 mg/kg thus had neither a positive nor a negative effect on the sensory profile of the fillets. Interestingly, a wet-feather/sweaty/barnyard aroma was significantly more prominent in the negative control meat in comparison to the positive control meat. It was speculated that this off-odour was as a result of a volatile organic compound (VOC), such as 4-ethylphenol, which may have been more prominent in the negative control

due to oxidative processes. Oxidation may have also played a role in the positive associations between the wet-feather attributes and thaw loss and initial juiciness.

Further research is recommended to investigate this phytogenic additive under different circumstances (i.e. in a sub-optimal environment in the presence of an intentional stressor) or at different inclusion levels; due to the overall lack of differences between the treatment groups and the negative control. The addition of other parameter measurements and techniques will also add value to future studies. Some such additions may include histomorphology studies of the GIT; investigation of blood constituents (i.e. lipid concentrations in the serum and antibody titer); evaluation of carcass fat content, bone density, mineral digestibility, and cortical and trabecular bone thickness; and methods to analyse and compare VOCs and fatty acid concentrations, as well as, meat and lipid oxidative rates.

As the VivoCare® product did not bring about any negative results throughout this study and it performed at a statistically similar level to the positive (AGP-included) control, it can be said that this product may have promising potential as an alternative for AGPs. It must be noted, however, that this statement is only relating to broilers raised under favourable environmental conditions, and that much research is still needed with various other conditions to adequately support this statement as a whole.